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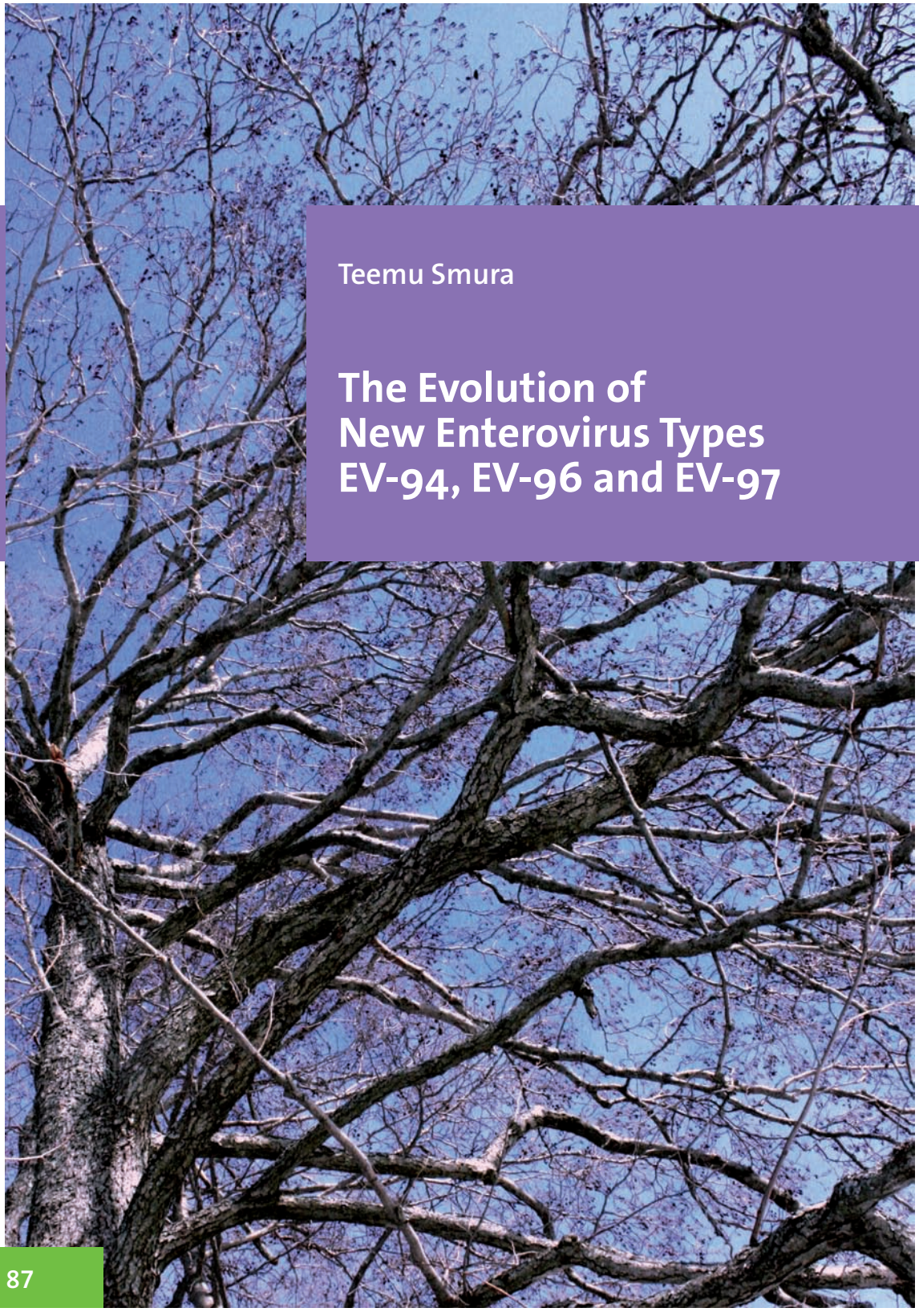

RESEARCH

Teemu Smura

The Evolution of New Enterovirus Types EV-94, EV-96 and EV-97

87

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ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki for public examination in the auditorium 1041 of Biocenter 2, Viikinkaari 5, Helsinki on September 5th 2012, at 12 o'clock noon

National Institute for Health and Welfare
Department of Infectious Disease Surveillance and Control
Virology Unit

University of Helsinki
Faculty of Biological and Environmental Sciences
Department of Biosciences

Helsinki 2012

NATIONAL INSTITUTE
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Cover photo: Teemu Smura, A non-phylogenetic tree constructed by mutation followed by natural selection and genetic drift.

ISBN 978-952-245-700-4 (printed)
ISSN 1798-0054 (printed)
ISBN 978-952-245-701-1 (pdf)
ISSN 1798-0062 (pdf)

Juvenes Print
Tampere, Finland 2012

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Dedicated to a dwarf with a pink hat

Abstract

Teemu Smura. The Evolution of New Enterovirus Types EV-94, EV-96 and EV-97. National Institute for Health and Welfare (THL). Research 87. 160 pages. Helsinki, Finland 2012.
ISBN 978-952-245-700-4 (printed); ISBN 978-952-245-701-1 (pdf)

Enteroviruses (genus *Enterovirus*, family *Picornaviridae*) are small single-stranded RNA viruses associated with a wide variety of acute and chronic diseases. The genus *Enterovirus* consists of ten species. Seven of the species, *Human enterovirus A* (HEV-A), HEV-B, HEV-C, HEV-D, *Human rhinovirus A* (HRV-A), HRV-B and HRV-C, cause infections in humans. The enterovirus species are further classified into serotypes (or types) by their antigenic properties and/or, more recently, their sequence similarities.

In this thesis, the genetic evidence for the classification of three new enterovirus types, enterovirus 94 (EV-94), EV-96 and EV-97, was presented. Further characterisation of prevalence and cell tropism was conducted for one of the new types, EV-94. Sequence analysis methods were used to infer evolutionary relationships between the new and previously known EV types and possible mechanisms behind the emergence of new enterovirus clades.

The enterovirus strains characterized in this thesis formed distinct monophyletic clades within the species HEV-B, HEV-C and HEV-D on the basis of their capsid-coding regions. These strains had less than 75% nucleotide (< 85% amino acid) sequence similarities with previously known enteroviruses in their VP1 protein coding sequences, suggesting that these strains represent previously unknown enterovirus types, designated as EV-94 (HEV-D), EV-96 (HEV-C) and EV-97 (HEV-B). The complete genome analysis of the EV-96 and EV-97 strains together with the previously characterised HEV-C and HEV-B strains suggested multiple inter-typic recombination events within non-coding and non-structural protein-coding regions of their genomes. No evidence of recombination was detected for EV-94 and other members of the HEV-D species. Furthermore, an apparently rare inter-species recombination event between the recently described HEV-A types (EV-90 and EV-91) and a member of the HEV-C/-D species was found at 5' untranslated region (5'UTR) of the genome.

EV-94 had unusually wide cell type and host species tropism *in vitro* and, despite recent discovery, a high prevalence of antibodies against EV-94 was detected among the Finnish population during the past three decades. These results indicate that this virus is highly prevalent and capable of invading a wide variety of tissues.

The sequence analysis presented in this thesis suggested different evolutionary patterns within enterovirus types (leading to intra-typic lineages) and evolution leading to larger scale (type-specific) differences. Intra-typic genetic change was dominated by silent mutations and amino acid polymorphism at immunogenic

sites. Inter-typic comparisons, however, suggested type-specific fixation of amino acids and differences in the location of polymorphic amino acid sites between EV types. These changes were proposed to be accompanied by structural alterations in the capsid proteins, possibly as a result of adaptation to larger scale changes in the environment.

Keywords: enterovirus, evolution, recombination, selection, adaptation, Human enterovirus A, Human enterovirus B, Human enterovirus C, Human enterovirus D, coxsackievirus, echovirus, enterovirus 94, enterovirus 96, enterovirus 97.

Tiivistelmä

Teemu Smura. Evolution of New Enterovirus Types EV-94, EV-96 and EV-97 [Uusien enterovirustyyppien EV-94, EV-96 ja EV-97 evoluutio]. Terveyden ja hyvinvoinnin laitos (THL). Tutkimus 87. 160 sivua. Helsinki, Finland 2012.
ISBN 978-952-245-700-4 (painettu); ISBN 978-952-245-701-1 (pdf)

Pikornavirusten heimoon kuuluvat enterovirukset ovat pieniä vaipattomia RNA-viruksia. Enterovirukset aiheuttavat monia akuutteja ja kroonisia tauteja. *Enterovirus*-suvussa on kymmenen lajia, joista seitsemän *Human enterovirus A* (HEV-A), HEV-B, HEV-C, HEV-D, *Human rhinovirus A* (HRV-A), HRV-B ja HRV-C aiheuttaa infektioita ihmisessä. Enterovirus lajit jaotellaan serotyypeiksi (tai tyypeiksi) niiden antigeenisten ominaisuuksien ja nykyisin myös perimän samankaltaisuuden perusteella.

Tässä väitöskirjassa tunnistettiin ja luokiteltiin kolme aiemmin tuntematonta enterovirustyyppiä, enterovirus 94 (EV-94), EV-96 ja EV-97, perimän nukleotidijärjestyksen perusteella. Uusien enterovirusryhmien mahdollista syntytapaa ja enterovirustyyppien välistä kehityshistoriaa tutkittiin vertailemalla virusten nukleotidi- ja aminohappojärjestyksiä. Yhden uuden enterovirustyypin (EV-94) esiintyvyyttä ja soluhakuisuutta tutkittiin tarkemmin.

Väitöskirjassa tunnistetut virukset kuuluivat nukleotidi- ja aminohappojärjestyksensä perusteella HEV-B, HEV-C ja HEV-D lajeihin. Niiden VP1 kuoriproteiinia koodaavat nukleotidisekvenssit olivat alle 75 % ja vastaavat aminohappojärjestykset alle 85 % samankaltaisia aikaisemmin tunnettuihin enterovirustyyppeihin verrattuna. Tämän perusteella virukset luokiteltiin uusiksi enterovirustyypeiksi ja nimettiin EV-94:ksi (HEV-D), EV-96:ksi (HEV-C) ja EV-97:ksi (HEV-B). EV-96 ja EV-97 kantojen genomeissa havaittiin merkkejä useista enterovirustyyppien välisistä rekombinaatioista. EV-94:llä ja muilla HEV-D lajin viruksilla rekombinaatiota ei havaittu. HEV-A lajiin kuuluvilla EV-90 ja EV-91 tyypeillä havaittiin harvinainen lajien välinen rekombinaatio genomin 5’ päässä.

EV-94:llä havaittiin muihin enteroviruksiin verrattuna poikkeuksellisen laaja kyky infektoida erilaisia solutyyppejä *in vitro*. Vasta-aineita EV-94:lle löydettiin lähes 80 %:sta kolmen vuosikymmenen aikana kerätyistä seeruminäytteistä. Näiden tulosten perusteella EV-94 on yleinen virustyyppi, joka pystyy lisääntymään monentyyppisissä kudoksissa.

Sekvenssianalyysin perusteella enterovirustyypin sisällä tapahtuva evoluutio on erilaista kuin eri virustyyppien kehittymiseen johtanut evoluutio. Enterovirustyypin sisällä tapahtuu enimmäkseen mutaatioita, jotka eivät johda aminohappomuutoksiin. Aminohappojärjestykseen vaikuttavia mutaatiota tapahtuu ainoastaan tietyissä, todennäköisesti antigeenisissa, kohdissa. Eri virustyyppien kehittyminen on puolestaan johtanut tiettyjen aminohappojen fiksoitumiseen erilaisiksi eri enterovirustyypeissä sekä polymorfisten aminohappoalueitten erilaiseen sijoittumiseen

eri virustyypeillä. Tällaiset muutokset saattavat johtua viruksen sopeutumisesta erilaisiin ympäristöihin ja ovat todennäköisesti myös johtaneet viruksen kapsidiproteiinien rakenteellisiin muutoksiin.

Contents

List of original papers

This thesis is based on the following articles which are referred to in the text by their roman numerals. Thesis also contains unpublished data.

I Smura T., Junttila N., Blomqvist S., Norder H., Kaijalainen S., Paananen A., Magnius L., Hovi T. & Roivainen M. (2007): Enterovirus 94, a proposed new serotype in human enterovirus species D. Journal of General Virology, 88: 849-858.

II Smura T., Blomqvist S., Paananen A., Vuorinen T., Sobotová Z., Bubovica V., Ivanova O., Hovi T. & Roivainen M. (2007): Enterovirus surveillance reveals proposed new serotypes and provides new insight into enterovirus 5'-untranslated region evolution. Journal of General Virology, 88: 2520-6.

III Smura T., Blomqvist S., Hovi T. & Roivainen M. (2009): The complete genome sequences for a novel enterovirus type, enterovirus 96, reflect multiple recombinations. Archives of Virology, 154:1157-61.

IV Smura, T., Ylipaasto, P., Klemola P., Kaijalainen, S., Kyllönen L., Sordi V., Piemonti, L. & Roivainen M. (2010): Cellular tropism of Human enterovirus D species serotypes EV-94, EV-70 and EV-68: Implications for the pathogenesis. Journal of Medical Virology. 82: 1940-9.

V Smura, T., Savolainen-Kopra, C., Blomqvist, S., Hovi, T., Roivainen, M.: The roles of selection and recombination in the evolution of Human enterovirus C species subcluster containing EV-96, EV-99, CVA-21 and CVA-24. Manuscript.

Abbreviations

A	adenine
aa	amino acid
AFP	acute flaccid paralysis
AHC	acute haemorrhagic conjunctivitis
ARI	acute respiratory infection
C	cytosine
Ca^{2+}	calsium
CD	cluster of differentiation
CPE	cytopathic effect
cre	cis-acting replication element
CVA	Coxsackievirus A
CVB	Coxsackievirus B
DAF	decay-accelerating factor
E	echovirus
eIF	eukaryotic initiation factor
EV	enterovirus
FBS	fetal bovine serum
G	guanine
HCAR	human Coxsackievirus and adenovirus receptor
HEV	human enterovirus
HMFD	hand-foot-mouth disease

HRV	human rhinovirus
ICAM	intercellular adhesion molecule
IRES	internal ribosomal entry site
ITAF	IRES trans-activating factor
La	lupus autoantigen
nt	nucleotide
ORF	open reading frame
PABP	poly(A)-binding protein
PCBP2	poly(rC)binding protein 2
p.i.	post infection
PTB	polypyrimidine tract-binding protein
PV	poliovirus
PVR	poliovirus receptor
RdRp	RNA dependent RNA-polymerase
RES	reticuloendothelial system
RNA	ribonucleic acid
SVDV	swine vesicular disease virus
T	thymine
$TCID_{50}$	50 % tissue culture infectious dose
Unr	upstream of N-ras protein
UTR	untranslated region
Vpg	viral protein genome-linked

1 Introduction

Enteroviruses (genus *Enterovirus*, family *Picornaviridae*) are small positive-stranded RNA viruses with icosahedral capsid structure. The genus *Enterovirus* consists of ten species. Seven of the species, *Human enterovirus A* (HEV-A), HEV-B, HEV-C, HEV-D, *Human rhinovirus A* (HRV-A), HRV-B and HRV-C, cause infections in humans. All of the enterovirus species are further classified to serotypes (or types) by their antigenic properties and/or, more recently, their sequence similarities. In this thesis, a vernacular term 'enterovirus' is used for the members of the HEV-A, -B, -C and -D species, whereas the term 'genus *Enterovirus*' refers to the members of all four HEV species and three HRV species.

Enteroviruses are among the most common viruses worldwide. Although a large majority of enterovirus infections are asymptomatic, these infections are known to induce a wide variety of diseases, both acute and chronic. Enteroviruses use faecal-oral and respiratory routes of transmission, and the primary replication site is in the mucosa of the respiratory or gastro-intestinal tracts. Occasionally, the virus may spread via the lymphatic system and circulation to secondary targets, including the central nervous system, heart and pancreas.

To prevent diseases stemming from virus infections, the epidemiology (dynamic distribution patterns) and pathogenesis (disease-causing mechanisms) of virus infections need to be understood. Evolutionary studies provide the keys for understanding both of these areas. RNA viruses have the capacity to evolve extremely fast due to their short generation times and high mutation rates. These characteristics provide the viruses with the ability to adapt rapidly to changes in the environment. For example, this rapid adaptation affects virus transmission among host populations (e.g., producing mutations resulting in viral epidemics or inter-species transmission) and virus distribution within the individual host (e.g., producing mutations resulting in changes in cell tropism or course of infection), which significantly contribute to the epidemiology and pathogenesis of virus infections. Due to frequent subclinical infections, for most enterovirus types, the genetic and phenotypic diversity, epidemiology and association with diseases are known only fragmentarily. Likewise, despite years of intensive research, the factors determining the pathogenesis of enterovirus infections remain enigmatic. This thesis addresses several important aspects of enterovirus biology, including sequence diversity, evolutionary patterns and viral cell tropism - a consequence of sequence diversity and evolution.

2 Review of the Literature

2.1 Enterovirus structure

The three dimensional structure of several enterovirus types has been determined, including poliovirus 1 (PV-1) (Hogle et al., 1985), PV-3 (Filman et al., 1989), echovirus 1 (E-1) (Filman et al., 1998), E-7 (He et al., 2002, Plevka et al., 2010), E-11 (Stuart et al., 2002), coxsackievirus A9 (CVA-9) (Hendry et al., 1999) coxsackievirus B 3 (CVB-3) (Muckelbauer et al., 1995), swine vesicular disease virus (SVDV - a lineage of CBV-5) (Fry et al., 2003, Verdaguer et al., 2003) and enterovirus 71 (EV-71) (Plevka et al., 2012). All of these enterovirus types show strikingly similar features.

The external diameter of EV particles is approximately 30 nm. The capsid of the virus is composed of 60 protomers that form an icosahedral lattice with five-fold and three-fold symmetry axes (Fig. 1c). Each protomer contains one copy of the viral capsid proteins VP1-VP4, which share a common wedge-like shape composed of a β-barrel structure with eight beta strands (named B to F) that form two antiparallel (BIDG and CHEF) β-sheets (Fig. 1b). The narrow ends of the VP1 β-barrels cluster around the five-fold axes of viral particle and the narrow ends of VP2 and VP3 β-barrels alternate around the three-fold axes of the viral particle (Filman et al., 1989). VP4 and the amino (N-) termini of VP1, VP2 and VP3 are inside the capsid and contribute to the stability of the virion via a network of protein-protein contacts. The C-termini and connecting loops between the β -sheets of VP1, VP2 and VP3 are at the surface of the virion.

The distinctive features of the capsid surface include a star-shaped plateau at the five-fold axes of symmetry with a depression (‘canyon’) around the axes, a three-bladed propeller-like structure at the three-fold axes of symmetry and a depression that crosses the two-fold axes (Rossmann et al., 1985) (Fig. 1a). Some but not all virus-receptor interaction sites are located at the canyon (Belnap et al., 2000, Stuart et al., 2002, Xiao et al., 2005, Zhang et al., 2008).

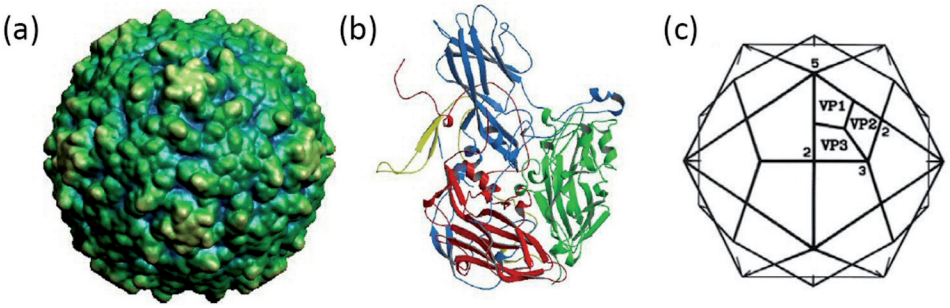


Figure 1. The structure of the enterovirus capsid (modified from Carrillo-Tripp et al., 2009, Filman et al., 1989). (a) The surface of the PV-1-Mahoney capsid. (b) A ribbon diagram that represents the PV-1-Mahoney protomer structure. VP1 is shown in blue, VP2 in green, VP3 in red and VP4 in yellow. (c) A schematic representation of the icosahedral capsid structure.

2.2 Enterovirus genome

The enterovirus genome contains approximately 7500 nucleotides. The genome has a single open reading frame (ORF) flanked by 5' and 3' untranslated (or non-coding) regions (UTR or NCR) and a poly(A) tail (Kitamura et al., 1981) (Fig. 2). The genome is covalently linked to the VPg protein at the 5' end. The non-coding regions of the genome contain secondary structures essential for replication and translation. The 'clover-leaf' structure at the 5'UTR (Andino et al., 1990, Andino et al., 1993, Rohll et al., 1994) and the stem-loop structures at the 3'UTR (Jacobson et al., 1993, Melchers et al., 1997, Mirmomeni et al., 1997, Pilipenko et al., 1992, Pilipenko et al., 1996) are required for viral replication and a highly ordered internal ribosomal entry site (IRES) structure at the 5'UTR is required for the initiation of translation (Chen & Sarnow, 1995, Molla et al., 1992, Pelletier & Sonenberg, 1988).

The ORF is translated into single polypeptide that is subsequently self-cleaved by viral proteases (reviewed in Palmenberg, 1990) (Fig. 2). The coding sequence can be divided into the three regions, P1, P2 and P3, according to the respective encoded proteins (see chapter 2.3.2). The coding region also contains cis-acting replication element (cre), a secondary structure within the 2C-coding region (Cordey et al., 2008, Goodfellow et al., 2000, van Ooij et al., 2006) that is involved in VPg uridylation during genome replication (see chapter 2.3.2) (Paul et al., 2000, Rieder et al., 2000).

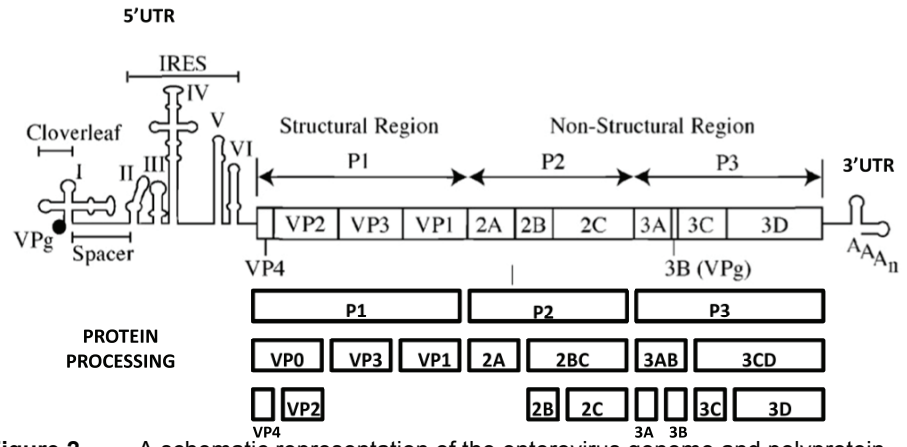


Figure 2. A schematic representation of the enterovirus genome and polyprotein processing (modified from De Jesus., 2007).

2.3 Enterovirus life cycle

Enterovirus infection begins with the attachment of a virus to a cell surface receptor. This attachment leads to changes in the capsid structure and release of the viral genome into the cell. The viral genome is translated by cellular ribosomes to single polypeptides that are self-cleaved to yield viral proteins, including viral RNA-dependent RNA polymerase, which is a protein required for viral replication. The replication begins with the synthesis of negative strand RNA that is subsequently used as a template for positive strand RNA synthesis. The positive strand genomes assemble with capsid proteins to form mature virions. Finally, the cell is lysed, which releases the virions. The complete life cycle of enteroviruses typically takes 5 to 10 hours.

2.3.1 Enterovirus-receptor interaction

A wide variety of cell attachment receptors have been found for enteroviruses. A virus can use a single receptor for attachment and entry or several different receptors and co-receptors during infection (Rossmann et al., 2002). Furthermore, a virus may utilise different receptors when infecting different cell types. Different viruses may use the same receptor, often with different binding sites (receptor footprints). For example, EV-70 uses the decay accelerating factor (DAF, also called CD55) in HeLa cells (Karnauchow et al., 1996) and sialic acid-containing molecules in leukocytes (Alexander & Dimock., 2002, Haddad et al., 2004, Nokhbeh et al., 2005). CVB-3, E-7 and E-12 bind to DAF but with different receptor footprints (Bergelson et al., 1994, Bergelson et al., 1995, Bergelson et al., 1997, Bhella et al., 2004, Plevka et al., 2010).

After attachment to the receptor the viral capsid must dissociate in order to release the viral genome into the cell. The uncoating events may differ between enteroviruses. For the enterovirus types that have their receptor binding site at the viral capsid canyon (e.g., PV, CVB, CVA-21), it has been suggested that the receptor binding releases a 'pocket factor', which is a fatty acid like-ligand that is bound to a hydrophobic pocket underneath the canyon floor. This release leads to the destabilation of the virus capsid and externalisation of myristoylated VP4 and the hydrophobic N-termini of VP1 (Chow et al., 1987, Chow et al., 1987, Crowell & Philipson, 1971, Fricks & Hogle, 1990, Huang et al., 2000) that is inserted into the cellular membrane (Danthi et al., 2003). These events lead to the formation of a pore through which the viral RNA may pass into the cell (Tosteson & Chow, 1997, Tosteson et al., 2004). The entry mechanisms of enteroviruses are currently under intensive research (reviewed in Marsh & Helenius, 2006, Pietiainen et al., 2005). So far, several putative entry mechanisms have been described (e.g., clathrin-dependent and clathrin-independent, lipid raft-dependent and dynamin-dependent caveolar mechanisms). Furthermore, the entry mechanism may vary from one cell type to another (Brandenburg et al., 2007, Coyne et al., 2007).

2.3.2 Translation

The viral RNA has to be translated before replication because the host cells lack RNA-dependent RNA polymerases (RdRp) required for viral replication, and viral enzymes are not brought into the cell by the virus. The virus genome lacks the 5'-terminal cap structure that is a translation initiation factor assembly site in eukaryotic cells. Instead, the 5' non-coding region of the virus genome contains a highly conserved secondary structure (internal ribosome entry site (IRES)) that promotes translation initiation by internal ribosome binding (reviewed in Bonderoff & Lloyd, 2008).

Picornavirus IRES structures are classified into two major groups: type I (enteroviruses and rhinoviruses) and type II (aphtoviruses and cardioviruses). The type I IRES (based on PV) contains five stem-loop regions (Fig. 2) (Le & Zuker., 1990, Pilipenko et al., 1989, Poyry et al., 1992, Rivera et al., 1988, Skinner et al., 1989). The recruitment and binding of the ribosomal subunits to the IRES is mediated by several canonical translation initiation factors that are also used by cellular cap-dependent translation machinery, and non-canonical translation factors that are not involved in eukaryotic cap-dependent translation (IRES trans-activating factors (ITAFs)). For poliovirus, the canonical translation factors include eukaryotic initiation factor 4 (eIF4) family members, eIF1, eIF1a, eIF5 and eIF5b, and the non-canonical translation factors include polypyrimidine tract-binding protein (PTB), poly(rC)binding protein 2 (PCBP2), upstream of N-ras (Unr) and lupus autoantigen (La) (reviewed in Bonderoff & Lloyd, 2008). ITAFs may stabilise the structure of the IRES to a conformation that is suitable for the binding of canoni-

cal translation factors and ribosomal subunits (Pilipenko et al., 2000). In addition to the 5'UTR binding proteins, the 3'UTR poly(A)-binding protein (PABP) enhances IRES-mediated translation (Imataka et al., 1998) via inter-actions with the IRES binding translation initiation factors.

The open reading frame of the enterovirus genome is translated to a single polyprotein of ~250 kDa. During translation, the polyprotein is self-cleaved (in cis) by viral proteinase 2A to form P1 and proteinase 3C to form P2 and P3 (Fig. 2). The primary cleavage is followed by a secondary cleavage (in cis or trans) to form mature proteins (reviewed in Sean & Semler, 2008). P1 contains viral capsid proteins (VP4-VP1), whereas P2 and P3 contain non-structural proteins involved in protein processing, shut-off of host cell translation, host cell membrane rearrangement and RNA replication.

Because IRES-mediated translation is intrinsically less efficient than cap-dependent translation, the virus has to inhibit host translation to ensure that the viral RNA does not have to compete with the host mRNA for translation initiation factors. This so-called host shut-off is mediated by the cleavage of a subset of translation initiation factors. Viral 2A proteinase cleaves eIF4GI (Zamora et al., 2002) and eIFGII (Gradi et al., 1998), and 2A and 3C proteinases cleave PABP (Joachims et al., 1999, Kerekatte et al., 1999, Kuyumcu-Martinez et al., 2004), thereby inhibiting cellular (cap-dependent) translation but enhancing viral IRES-mediated translation (through an unknown mechanism) (Hambidge & Sarnow, 1992).

Eventually, viral translation has to be inhibited to use the viral genome as a template for replication. The accumulation of viral non-structural proteins may induce the transition from viral translation to replication. This transition may occur by the binding of viral 3CD protein to the clover-leaf, thereby increasing the affinity of PCBP2 for this element and decreasing the availability of PCBP2 for IRES-mediated translation (Gamarnik & Andino., 2000). Several ITAFs are also cleaved by viral 3C proteinase, which results in the inhibition of viral translation (Back et al., 2002, Perera et al., 2007).

2.3.3 Replication

Following the translation of the viral genome, the enterovirus RNA is translocated to the membranous vesicles, where the synthesis of negative and positive strands occurs. The mechanism of translocation is not known, but it may involve viral 3AB and 2C proteins. 3AB has been shown to interact with membranous vesicles and viral proteins involved in replication (Towner et al., 1996), whereas 2C has been shown to interact with membranous vesicles and negative-stranded RNA (Banerjee et al., 1997, Echeverri & Dasgupta, 1995).

The replication of the enterovirus genome occurs in two steps: first, a negative (complementary) strand is synthesised from the parental positive strand RNA, and

the negative RNA strand is then used as a template for the synthesis of daughter positive strand RNA molecules that can be used for translation or packaged into virions (reviewed in Sean & Semler, 2008).

The initiation of negative strand synthesis involves the interactions of viral and cellular proteins in the 3' and 5'UTRs of the viral genome. A so-called ternary complex, composed of PCBP, 3CD and a clover-leaf structure at the 5'UTR of the viral genome, circularise the template RNA by binding with PABP in the 3' poly(A), thereby bringing the replication complex to the 3' poly(A) tract for the initiation of negative strand replication (Barton et al., 2001, Bell et al., 1999, Gamarnik & Andino, 1997, Herold & Andino, 2001, Parsley et al., 1997). In addition to RNA-dependent RNA polymerase 3D, the replication complex contains other viral and host macromolecules (including 2C and possibly 3AB, 3CD and hnRNPC (Sean & Semler, 2008)). At the 3' poly(A) tract, the 3D polymerase uridylylates the Vpg protein (Paul et al., 1998) and forms VpgpUpU, which is used as a primer (complementary to poly(A)) for negative strand synthesis (Flanegan & Baltimore, 1977, Yogo & Wimmer, 1973).

The initiation of positive strand synthesis begins with the recruitment of the replication complex containing uridylylated-Vpg to the clover-leaf structure at the 3'end of the negative-strand RNA (i.e., complementary to the 5' clover-leaf of the positive-strand RNA) (Andino et al., 1990). During the initiation of positive strand synthesis, the uridylylation of Vpg occurs on the cre (cis-acting replicative element) stem-loop structure at the 2C coding region of viral RNA, where the 3D polymerase catalyses the formation of a phosphodiester bond between the tyrosine of Vpg and a uridine residue using the adenosine residue of cre as a template (Paul et al., 2003). VpgpUpU anneals with the two conserved adenylate residues at the 3' end of the negative strand (Sharma et al., 2005). During positive strand RNA synthesis, multiple RNA strands are simultaneously synthesised from a negative strand template. This simultaneous synthesis is possible, because the hybridisation of the nascent positive strand RNA with the negative template RNA is most likely inhibited by the extensive secondary structure formation in the positive strand RNA or the helicase activity of 3D (Cho et al., 1993) or 3AB (DeStefano & Titilope, 2006).

2.3.4 Virus assembly and release

Enterovirus assembly and release apparently do not require host cell components, and proceed independently. Following polyprotein cleavage, VP1, VP3 and myristoylated immature capsid protein VP0 (consisting of VP4 and VP2) spontaneously assemble into protomers and further into pentamers of five protomers. The pentamers assemble into empty capsids that contain 60 copies of each protein. It is not known whether the viral RNA is encapsidated by capsid protein pentamers (Nugent & Kirkegaard, 1995) or insertion into preformed empty capsids (Jacobson

& Baltimore, 1968). Either way, the encapsidation is functionally coupled to RNA replication because only newly synthesized virus RNA is packaged to provirion (Molla et al., 1991, Nugent et al., 1999). During the encapsidation, VP0 is autocatalytically cleaved into VP4 and VP2. This cleavage stabilises the virion and increases its infectivity by establishing the ordered N-terminal network of capsid proteins (see chapter 2.1).

The mechanism of virion release from the host cell is not clear. Typically, infected cells develop characteristic morphologic changes (including condensation of chromatin, nuclear blebbing, proliferation of membranous vesicles, leakage of intracellular components, visible rounding and then shrivelling of the cell), which is known as the cytopathic effect (CPE) (Racaniello, 2007). The virus-induced disruption of Ca^{2+} homeostasis (by increasing membrane permeability to Ca^{2+}) may lead to a cascade that results in a loss of cell membrane integrity, necrosis and enhanced virus progeny release (Bozym et al., 2011, van Kuppeveld et al., 1997). The details of the process, however, most likely depend on the cell type and virus strain involved.

2.4 Pathogenesis of enterovirus infection

2.4.1 The course of infection

Enteroviruses are transmitted *via* the faecal-oral and respiratory routes. The primary site of infection is at the mucosa of the respiratory or alimentary tracts. Most of the human enteroviruses (contrary to human rhinoviruses) are resistant to the acidic environment of the stomach and therefore capable of accessing the small intestine, where virus replication can continue for several (2-8) weeks. During this period, the progeny virus is excreted in the faeces. The cell type where primary replication of the virus occurs is not known, but poliovirus might be able cross the epithelial barrier *via* M-cells in Peyer's patches (Ouzilou et al., 2002, Sicinski et al., 1990) and enteroviruses can generally be isolated from the lymphatic tissues of the gastrointestinal tract, such as the tonsils, Peyer's patches of the ileum and mesenteric lymph nodes (reviewed in Mueller et al., 2005). Despite entering and replicating in the lymphatic tissues, the virus shedding in the intestinal lumen is most likely preceded by an infection of the intestinal epithelial cells from the basolateral surface (reviewed in Pfeiffer, 2010).

Focal virus multiplication in the gut mucosa may lead to transient 'minor' viremia, during which the virus spreads through the lymphatic system and circulation and may lead to virus replication in the reticuloendothelial system (RES), which includes phagocytic cells, e.g., mononuclear leukocytes accumulated in the lymph nodes and spleen. This replication may amplify the infection to 'major' viremia and increase the probability of secondary target tissue infection (Freistadt & Eberle, 1996). The secondary target tissues vary by enterovirus strain but in-

clude the central nervous system, meninges, heart and pancreas. Most enterovirus-related diseases are consequences stemming from the viral infection of secondary target tissues. However, in the case of poliovirus, only a fraction of the infected individuals develop disease symptoms, and poliomyelitis affects less than 1% of PV-infected individuals (reviewed in Mueller et al., 2005).

2.4.2 Cell and tissue tropism

Factors affecting enterovirus cell and tissue tropism include receptor specificity, the presence of host cell factors required for viral translation and replication, the host immune response and the cell-cycle stage of the host cell (reviewed in Whitton et al., 2005). Cell surface receptors are needed for virus attachment, entry and (in the case of poliovirus) uncoating. Although required for infection, mere receptor expression does not predict tissue tropism *in vivo*. For example, poliovirus receptor (PVR) is expressed at high levels in the liver, lung and heart, but these organs are not thought to be target tissues for poliovirus replication. The viral IRES structure appears to be an important tissue tropism determinant for polioviruses and Coxsackie B viruses (Dunn et al., 2000, Dunn et al., 2003, Evans et al., 1985, Gromeier et al., 1996, La Monica & Racaniello, 1989, Svitkin et al., 1985), which is most likely due to interactions with host cell translation initiation factors, the expression levels of which are cell type-specific (Guest et al., 2004, Pilipenko et al., 2001, reviewed in Bonderoff & Lloyd, 2008). The host innate immune response also contributes to viral cell tropism, because the type I interferon response has been shown to restrict poliovirus and coxsackievirus replication in non-target tissues (Flodstrom et al., 2002, Ida-Hosonuma et al., 2005). Furthermore, enteroviruses might establish productive infections in cells at the G1/S phases of the cell cycle (i.e., during cellular growth and DNA replication) and persistent/latent infections in quiescent cells (reviewed in Feuer & Whitton, 2008). Highly active cell types would therefore be most susceptible to enterovirus infections.

2.4.3 Diseases

Although the large majority of the infections are asymptomatic, remarkably diverse disease manifestations have been associated with enteroviruses. These diseases include acute (e.g., respiratory tract infections, hand-foot-mouth disease (HMFD), acute haemorrhagic conjunctivitis (AHC), aseptic meningitis, encephalitis, acute flaccid paralysis (AFP), myocarditis, epidemic pleurodynia, herpangina) and chronic (e.g., poliomyelitis, dilated cardiomyopathy, type 1 diabetes, chronic fatigue syndrome) diseases (reviewed in Pallansch & Roos, 2001). The disease symptoms are generally not specific to a distinct enterovirus type, and several different virus types can induce similar disease manifestations. However, a single virus type can also cause several different diseases. Knowledge of the pathogenetic mechanisms of enteroviruses is incomplete, but direct cellular damage due to

lytic virus infection and immunopathogenetic mechanisms (including autoimmunity) has been implicated for many enterovirus-induced diseases.

The susceptibility to enterovirus infections and enterovirus-induced diseases vary by age group and gender. Males are more susceptible than females with male to female ratio of 1.2-2.5:1 (reviewed in Pallansch & Roos, 2001). The severity of enterovirus-induced diseases is generally considered to correlate with the age of the first infection of the given virus type; a delay in the age of the first infection increases the risk of more severe symptoms (reviewed in Pallansch & Roos, 2001).

2.5 Epidemiology of enterovirus infections

The epidemiological data for the majority of the enterovirus types are incomplete due to apparent pitfalls in the enterovirus surveillance data. Most infections are likely to remain unnoticed due to a mild course or lack of disease symptoms. Furthermore, the isolation of a virus from a patient does not prove a causal relationship between the virus and the symptoms. The most reliable data about disease incidence with viral etiology can be derived from prospective longitudinal surveillance of a defined population. However, prospective cohort studies are expensive and difficult to administrate; therefore, most data about enterovirus-related disease incidence are derived from a surveillance of disease cases. Sewage surveillance, which is a strategy adopted for poliovirus surveillance in many countries (including Finland), provides information about enterovirus prevalence (including asympotomatic carriers) in the population (Hovi., 2006) but does not allow inferences on disease associations. Therefore, epidemiological data about enteroviruses are highly biased due to sampling of certain symptoms (e.g., AFP), generally higher sampling frequencies in countries with high socio-economic status (and populations within the country), increased sampling during epidemics and methodological restrictions, such as culturing virus in a limited set of cell lines.

Despite the obvious biases, some generalisations about the epidemiology of enterovirus infections can be made. Enterovirus prevalences vary by species, type, geographic location and time. Among healthy children in countries with high socio-economy status, enteroviruses have been detected in approximately 5-15 % of stool samples collected during the first few years of life (Gamble., 1962, Patti et al., 2000, Simonen-Tikka et al., 2011, Tapia et al., 2011, Witso et al., 2006, Witso et al., 2010). Maternal antibodies are thought to protect infants for the first 4-6 months; after the first year of life, the enterovirus incidence appears to increase (Gamble., 1962, Witso et al., 2006, Simonen-Tikka et al., 2012), which coincides with the time children are exposed to the infections in kindergarten. Among the lower socio-economic populations, the exposure of infants to enteroviruses most likely occurs earlier. The highest amount and duration of virus shedding occur

during the first infection with a given virus type (because no specific antibodies and memory B-cells against this virus type are present).

Two climatic factors – high degree of humidity and elevated temperatures – apparently facilitate EV transmission. Correspondingly, in temperate climates enterovirus infections are most prevalent during the summer and early autumn, whereas in tropical climates enterovirus circulation tends to be year-round or associated with the rainy season (Pallansch & Roos, 2001).

In temperate climates HEV-B is generally considered to be the most prevalent enterovirus species. However, high prevalences of HEV-A types have also been reported among healthy children (Simonen-Tikka et al., 2011, Simonen-Tikka et al., 2012, Witso et al., 2006) and during certain epidemiological circumstances, such as the recent HFMD outbreak (Blomqvist et al., 2010a, Bracho et al., 2011, Mirand et al., 2012). It is not known whether the contradictory prevalence reports are due to methodological differences in detection sensitivities, biased sampling based on disease symptoms or actual changes in the prevalences of the HEV-A and HEV-B species over time. The HEV-C strains are rarely detected in temperate regions, whereas these strains are highly prevalent in tropical and subtropical regions (Arita et al., 2005, Jegouic et al., 2009, Rakoto-Andrianarivelo et al., 2005, Rakoto-Andrianarivelo et al., 2007). HEV-D is considered to be the least prevalent of enterovirus species worldwide. However, a high seroprevalence of neutralising antibodies against EV-68 and recent reports of EV-68 epidemics suggest that this type may be more prevalent than was previously thought (Blomqvist et al., 2002, Centers for Disease Control and Prevention (CDC)., 2011, Higgins., 1982, Ikeda et al., 2012, Imamura et al., 2011, Jacobson et al., 2012, Kaida et al., 2011, Meijer et al., 2012, Oberste et al., 2004, Rahamat-Langendoen et al., 2011, Schieble et al., 1967, Tokarz et al., 2011, IV). EV-68 is a respiratory pathogen (Oberste et al., 2004, Schieble et al., 1967) and the lack of detection has most likely been due to a sampling bias. Another HEV-D type, EV-70, was first detected in Ghana in 1969 (Mirkovic et al., 1973) and has since caused two acute haemorrhagic conjunctivitis pandemics (Kew et al., 1983, Kono., 1975, Palacios & Oberste, 2005) and several smaller scale epidemics (Bern et al., 1992, Gogate., 1997, Maitreyi et al., 1999, Shulman et al., 1997). However, no reports of EV-70 detection from the past decade exist, which suggests a low prevalence of this virus at the moment.

An enterovirus type may have an epidemic or endemic circulation pattern. Epidemic virus types (e.g., E-9, E-11, E-30 (Khetsuriani et al., 2006, Oberste et al., 2003, Savolainen et al., 2001)) show incidence peaks at certain years followed by periods of lower prevalence, whereas endemic virus types (e.g., CVA-9, CVB-2, CVB-4 (Khetsuriani et al., 2006, Mulders et al., 2000, Santti et al., 2000)) are typically isolated every year with little variation in the numbers of isolated strains. However, large scale epidemics from endemic virus types may occur occasionally, and lineages of epidemic virus types may circulate silently in a distinct geographi-

cal region for several years before causing an outbreak (Savolainen-Kopra et al., 2011, Tee et al., 2010). Furthermore, outbreaks may occur over large (e.g., E-30 and EV-71) or restricted (e.g., E-9) geographical areas, over short periods (E-9) or several years (E-11) and have a regular cyclic temporal pattern (E-9, E-30, EV-71) or irregular periods of quiescence (E-11). The epidemiological pattern of a virus type may also depend on the climate. Some of the enterovirus types that are epidemic in temperate climates are considered to be endemic in warmer climates (Apostol et al., 2012, Bahri et al., 2005). The lineages of some of the epidemic enterovirus types, such as E-30 (Bailly et al., 2009, Ke et al., 2011, Mirand et al., 2007, Oberste et al., 1999b, Palacios et al., 2002, Savolainen et al., 2001, Savolainen-Kopra et al., 2011), E-11 (Bouslama et al., 2007a, Savolainen-Kopra et al., 2009b) and EV-71 (Tee et al., 2010) have a ladder-like, chronological, phylogenetic structure that reflects emergence followed by extinction or dormancy and possible re-emergence of dominant lineages. Several lineages may also co-circulate during an outbreak (Leveque et al., 2010, McWilliam Leitch et al., 2010, Oberste et al., 2003, Savolainen-Kopra et al., 2009b).

2.6 Enterovirus evolution

Three mechanisms – mutation, recombination and, in the case of viruses with a segmented genome, reassortment – produce variation in RNA virus populations (reviewed in Domingo et al., 1996, Savolainen-Kopra & Blomqvist, 2010). Point mutations are minimal changes in the genome sequence. These mutations may occasionally have a profound effect on the phenotype. Recombination and reassortment, on the other hand, segregate RNA fragments between viruses, which transfers larger segments of genetic information. Therefore, mutation generates variation whereas recombination and reassortment shuffle this variation, producing new combinations of previously generated mutations.

Irrespective of the mechanism by which the variant genomes were produced, these genomes are subjected to genetic drift and natural selection that ultimately define whether a given mutation becomes fixed or eliminated or the corresponding position remains polymorphic in the population. Genetic drift is random change in the relative frequencies of gene variants (alleles) in the population. Genetic drift may have a strong effect on evolution, especially if the population size is very small on occasion. During the infectious cycle, enteroviruses are expected to go through several genetic bottle-neck events, where the virus population size decreases to a small fraction of the original, suggesting that random genetic drift may have a profound effect on the genetic structure of enterovirus populations. Selection, on the other hand, is a directional force driving evolution. Selection acts on mutations that change the fitness of an organism. Positive selection increases the frequency of advantageous gene variants, whereas deleterious (harmful) mutations encounter negative (purifying) selection that decreases their frequency.

2.6.1 Mutation – Causes and Consequences

Point mutations are introduced into the enterovirus genome primarily by errors made by the viral polymerase during replication and possibly by additional mechanisms such as mechanisms that involve host cell deaminating enzymes (reviewed in Duffy et al., 2008). The RNA polymerases of RNA viruses have generally very low fidelity and introduce mutations at a rate of 10^{-5} to 10^{-3} substitutions per nucleotide per replication event (Drake., 1993, Drake & Holland, 1999, Duffy et al., 2008, Holland et al., 1982). The limited replication fidelity of RNA-dependent RNA polymerase is due to the absence of a proof-reading/repair mechanism and postreplicative error correction mechanism. Secondary structures in the virus genome can further increase the error-frequency by increasing the chance of template slippage during replication (Pathak & Temin, 1992, Pita et al., 2007). As a result of high mutation frequency, enteroviruses form highly polymorphic populations (i.e., quasispecies) where most viral genomes differ from each other by one or more nucleotides.

A high mutation frequency has both harmful and beneficial consequences to viruses. Because all organisms are products of natural selection, most random non-synonymous mutations are likely to decrease the fitness of an organism. Asexually reproducing organisms with error-prone replication can therefore be expected to incorporate fitness-decreasing mutations into their genomes, unless compensatory mechanisms such as back mutation or recombination can restore the state of the genome (Muller, 1964). This phenomenon is known as 'Muller's ratchet'. Viruses with a small population size and high mutation rate are most likely affected by this mechanism. Thus, extremely heterogenic RNA virus populations are likely to contain genomes with fitness-decreasing mutations (Domingo et al., 1978).

The high mutation frequency is most likely beneficial when the virus needs to adapt to an environmental change or new environment. Due to a high mutation rate, a viral population contains a reservoir of potentially useful (pre-adapted) variants. Furthermore, the high mutation frequency provides a virus with the ability to efficiently explore the changing adaptive landscape (with local peaks of high fitness and valleys of low fitness).

Furthermore, the replication fidelity of a polymerase is also likely to be under selection pressure. The fidelity of the poliovirus RNA polymerase can be increased by a single amino acid change (Pfeiffer & Kirkegaard, 2003). The increased fidelity has been shown to be associated with a decrease in fitness *in vitro* and a less virulent phenotype *in vivo*, which is possibly due to slower replication and inability to adapt to different tissues (Pfeiffer & Kirkegaard, 2005, Vignuzzi et al., 2006). The mutation frequency can therefore be depicted as a trade-off between the adaptability and stability of (advantageous) genetic features. In static environments natural selection can be expected to favour high fidelity polymerases to reduce the frequency of deleterious mutations. However, enteroviruses are

likely to encounter several different and constantly changing environments during their transmission cycle (such as the host immune system, differences between host individuals, different cell types) and thus benefit from rapid adaptability. There may also be a trade-off between the speed and fidelity of replication and the cost for rapid replication could be increased error frequency (Elena & Sanjuan, 2005). The enterovirus 'life style' of explosive replication causing acute (lytic) infections in constantly changing environment could therefore have favoured speed and adaptability at the expense of the replication fidelity.

The upper limit for the mutation frequency is determined by error threshold. Above the error threshold, too many errors accumulate to produce viable offspring or to pass genetic information to the next generation. The size of the enterovirus genome may be limited by the high mutation frequency, because large genomes accumulate more mutations than small genomes (Duffy et al., 2008). At extremely high mutation frequencies (i.e., frequencies close to error threshold), RNA viruses have been suggested to follow quasispecies population dynamics (Domingo et al., 2008). According to the quasispecies model, because the high mutation frequency prohibits the accurate passing of genetic information to the progeny, the viruses form a cloud of related genotypes that constantly mutate to one another (i.e., individual viral genomes are linked by a mutational coupling). The replication success of a genotype would not depend only on its own replication success but on the success of the whole quasispecies cloud around it. Thus, natural selection would act on the entire quasispecies population rather than on individual genomes and the whole population would evolve as a single unit.

Due to high mutation frequency, a mutational robustness (the ability to retain constant phenotype despite genotypic change) may be favoured instead of high fitness. In an extreme case, a population with low fitness but high robustness may outcompete a population with high fitness but low robustness (i.e., a population occupying a high but narrow peak in the adaptive landscape) (Bull et al., 2005, Burch & Chao, 2000, Codoner et al., 2006, Wilke et al., 2001). Therefore, another way to compensate for a high mutation rate might be to occupy the flat regions of the fitness landscape (with possibility to mutational robustness) instead of the narrow fitness peaks (where random mutations are highly likely to cause a drastic decrease in fitness) (Codoner et al., 2006, Elena & Sanjuan, 2005, Sanjuan et al., 2007). Although experimental evidence shows that RNA viruses follow the quasispecies dynamics in certain conditions (Burch & Chao, 2000, Codoner et al., 2006), it has been debated whether the quasispecies dynamics occur in viruses in nature, where conditions may not be present in which the quasispecies theory is applicable (Holmes & Moya, 2002, Holmes, 2010, Jenkins et al., 2001).

2.6.2 Recombination

Genetic recombination is a widespread phenomenon among enteroviruses (reviewed in Lukashev, 2005). During recombination, a 'donor' nucleotide sequence is introduced into an 'acceptor' RNA molecule. Recombination produces a new chimeric RNA that contains genetic information from at least two different sources. Enteroviruses are generally thought to utilise homologous recombination, where the donor sequence replaces a homologous region of the acceptor sequence and the structure of the molecule is unchanged (Lai, 1992).

According to strand-switching or copy-choice model (Cooper et al., 1974), the viral RNA-dependent RNA polymerase complex switches the template from one RNA molecule to another during replication, which forms chimeric RNAs. During the negative strand synthesis, secondary structure elements or nucleotide misincorporations may slow down and/or stop the RNA polymerase (Pilipenko et al., 1995, Romanova et al., 1986, Tolskaya et al., 1987, White & Morris, 1995). Subsequently, the nascent RNA strain may dissociate from the original template and serve as a primer to resume elongation on another template, producing a recombinant genome. This model has robust experimental support (Duggal et al., 1997, Jarvis & Kirkegaard, 1992, Kirkegaard & Baltimore, 1986, Nagy & Bujarski, 1995, Nagy et al., 1998, Simon & Nagy, 1996). The non-replicative model for enterovirus recombination suggests a cleavage of RNA occurs, followed by cross-ligation in a transesterification reaction (Gmyl et al., 1999, Gmyl et al., 2003). Because sequence homology is not required for non-replicative recombination, this mechanism may promote the horizontal transfer of genetic material between distantly related RNA viruses (Gmyl et al., 2003).

Recombination may provide the rapid adoption and spread of advantageous traits or permit the elimination of detrimental mutations (reviewed in Agol, 2006, Simon-Loriere & Holmes, 2011, Worobey & Holmes, 1999). There are, however, also limitations with the occurrence of recombination. Recombination requires the co-infection of a certain cell with two distinct virus strains. Recombination is also constrained by sequence dissimilarity between potentially recombining genomes (Worobey & Holmes, 1999). Therefore tissue/cell tropism, prevalence and evolutionary relatedness affect the probability of recombination between given virus genotypes.

Among the enteroviruses, intra-typic and inter-typic recombination has been detected. Recombination usually occurs only between members of the same enterovirus species. Inter-species recombination is a very rare phenomenon that has occurred only a few times during enterovirus evolution (Santti et al., 1999, II). Recombination appears to occur frequently in the non-structural regions of the genome, whereas recombination seems to be less frequent within the capsid coding region. However, in polioviruses, recombination in the 3' end of the VP1 capsid protein coding region (Blomqvist et al., 2003, Blomqvist et al., 2010b, Dedep-

sidis et al., 2008, Liu et al., 2000, Martin et al., 2002) and in the VP2 protein coding gene (Kyriakopoulou et al., 2006) has been detected. A chimeric strain of CBV-4/CBV-3 with a recombination site within VP3 protein coding region has also been described (Bouslama et al., 2007b).

2.7 Enterovirus classification

An enterovirus species is defined as a polythetic class of phylogenetically related serotypes or strains that share a limited range of natural hosts and host cell receptors, a significant degree of compatibility in proteolytic processing, replication, encapsidation and genetic recombination, a greater than 70 % amino acid sequence identity in P1, 2C and 3CD proteins and a genome (G+C) base composition that varies no more than 2.5 % (Knowles et al., 2012). According to current classification, the genus *Enterovirus* contains 10 species. Seven of the species, *Human enterovirus A* (HEV-A), HEV-B, HEV-C, HEV-D, *Human rhinovirus A* (HRV-A), HRV-B and HRV-C, are known to infect humans (Fig. 3). The other species of the genus infect monkeys (*Simian enterovirus A* and a yet unclassified novel species consisting of types SV-6, EV-103 and EV-108 that were isolated from captive simians (Oberste et al., 2002, Oberste et al., 2007, Oberste et al., 2008)), bovines (*Bovine enterovirus*) and pigs (*Porcine enterovirus B*). The genus *Enterovirus* forms the *Picornaviridae* family together with the genera *Aphthovirus*, *Avihepatovirus*, *Cardiovirus*, *Erbovirus*, *Hepatovirus*, *Kobuvirus*, *Parechovirus*, *Sapelovirus*, *Senecavirus*, *Teschovirus* and *Tremovirus*. In addition, several unclassified picornaviruses that infect a variety of host species including mammals (e.g., human cosavirus and salivirus, seal picornavirus 1, sheep picornavirus 1 and bat kobu-like virus), avians (e.g., turdiviruses), reptiles (e.g., tortoise picornavirus 1) and fishes have been identified (Knowles, 2012). The *Picornaviridae* family is further classified to the order Picornavirales (Le Gall et al., 2008).

2.7.1 Enterovirus typing

Human enteroviruses were originally classified by pathogenic properties, which resulted in four groups: polioviruses (PV), coxsackieviruses A (CVA) and B (CVB) and echoviruses (E). The first clinical descriptions of poliomyelitis were made in the 19th century and poliovirus was isolated and proved to be a causative agent for poliomyelitis in 1908 by Landsteiner and Popper. Coxsackie A viruses were first characterised during a poliomyelitis epidemic in 1948 from the faeces of paralysed children. In contrast to polioviruses, which can only infect primates, these viruses caused flaccid paralysis in suckling mice (Dalldorf & Sickles., 1948, Dalldorf & Sickles., 1949). The first coxsackie B viruses were isolated in 1949 from aseptic meningitis cases (Melnick et al., 1949) and found to cause spastic paralysis and generalised infection of newborn mice. The development of methods for virus isolation in cell cultures enabled the detection of viruses that did not

replicate in experimental animals. Echoviruses were first isolated from the stools of asymptomatic individuals in 1951 (Robbins et al., 1951). These viruses were cytolytic in cell cultures but could not infect common experimental animals. Echoviruses were later shown to induce multiple diseases in humans.

Neutralisation tests showed that all of these virus groups consist of several different serotypes (Bodian et al., 1949, Sickles & Dalldorf., 1949). A serotype is considered to be an antigenically distinct group of viruses. A strain is considered to represent a new serotype, if it is not neutralised to a significant extent by antisera raised against previously characterised viruses and not able to induce significant levels of neutralising antibodies against these viruses.

It was soon discovered that an individual serotype could not be associated with a single disease but rather with a wide range of clinical manifestations. Additionally, it was found that a limited number of molecular differences between the strains of a given serotype may affect the pathogenicity of the virus in mice, thus confusing the classification of serotypes by pathogenic properties. Thereafter, the serologically distinct new enterovirus strains were merely numbered in the order of their identification (EV-68 to EV-71). Furthermore, when partial genome sequencing became available for enterovirus identification, the classification by pathogenic properties was not found to correlate with the evolutionary relationships of the virus strains (Hyypia et al., 1997, Poyry et al., 1996).

Likewise, there are several difficulties in the antigenic typing of enteroviruses. The antigenic sites of the circulating viruses are under a constant evolutionary selection pressure posed by the host immune system, which results in antigenic drift. Thus, some enterovirus strains represent the antigenic continuum within a given serotype, which leads to non-reciprocal neutralisation, e.g., the virus isolate may be neutralised poorly by the antiserum that neutralises the reference strain, but the antiserum made against the isolate may be able to neutralise both the isolate and reference virus. These antigenic variants are called the prime strains of a serotype. Extensive cross-reactivity was also detected between some serotypes, which were later reclassified on a phylogenetic basis (Brown et al., 2003).

Sequence relationships offer a more robust basis for classification than phenotypic characteristics, and recent molecular detection and identification methods have surrogated antigenic typing (reviewed in Nasri et al., 2007). Modern classification of the enterovirus types is based on sequence variation in the capsid protein VP1-coding region. The classification based on this region correlates with the classification made using antigenic properties (Oberste et al., 1999, Oberste et al., 2000). The strains belonging to the same serotype have > 75% nucleotide (nt) and > 85% amino acid (aa) sequence similarity of VP1-coding sequences whereas the strains of different serotypes have < 70% nt similarity (< 85% aa similarity) (Oberste et al., 1999a, Oberste et al., 1999). However, in some cases, additional sequence data are needed to clarify the phylogenetic position of a virus strain

(Brown et al., 2009). In this thesis, the term 'type' or 'EV-type' is used instead of 'serotype' to include all of the strains classified by antigenic characteristics or sequence similarities. Because of the high frequency of recombination, the members of a type are monophyletic (i.e., have a single common ancestor) only in the capsid-coding region of the genome; thus the type designation provides information about this region only.

Molecular typing of enteroviruses has several advantages over the traditional antigenic typing. In addition to viral diagnostics, molecular typing provides information for detailed epidemiological and evolutionary studies. Furthermore, this typing has enabled the characterisation of several antigenically untypable enterovirus strains (reviewed in Smura et al., 2011). In this thesis, sequence data were used for the characterisation of three previously unknown enterovirus types and the evolutionary processes behind the appearance of these types.

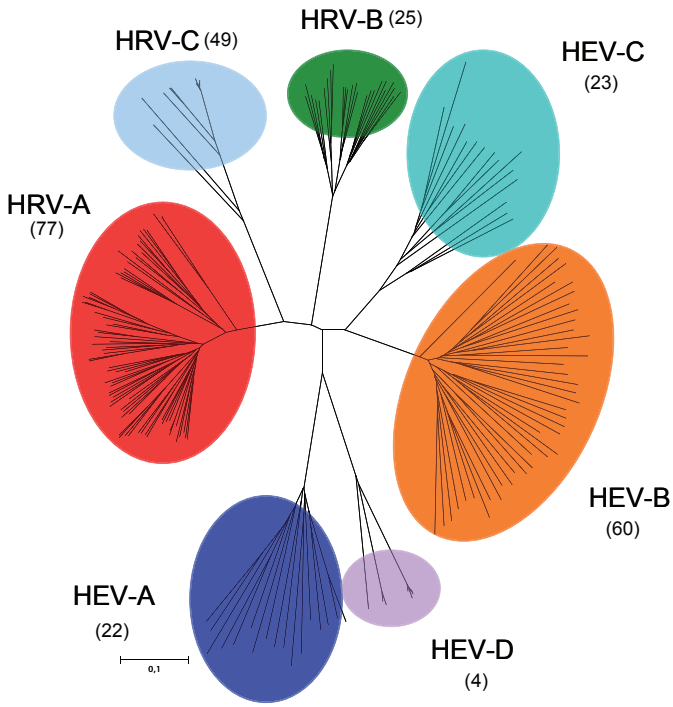


Figure 3. An unrooted phylogenetic tree constructed from VP1 protein-coding sequences using representatives of the seven enterovirus species capable of infecting humans. The number of currently known types in each species is given in parentheses.

3 Aims

The general aim of this thesis was to elucidate the processes behind the origin and appearance of novel enterovirus types.

The specific aims were the following:

1. To characterise enterovirus strains detected during enterovirus surveillance, which were 'untypable' with traditional methods (I-III).
2. To elucidate evolutionary relationships between the new and previously characterised enterovirus types and to assess the evolutionary patterns among the novel serotypes (I-III, V).
3. To detect experimentally phenotypic traits (e.g., cell tropism) that might affect evolution and pathogenetic properties of new enteroviruses (I & IV).

4 Material and Methods

4.1 Cell culture

The cell lines and primary cells used in the study are indicated in Table 1. The cell lines were maintained in a culture medium supplemented with 10% foetal bovine serum (FBS). The details of isolation and/or maintenance of the primary cells are included in the Material and Methods section of the respective original publication (IV).

4.2 Viruses

The virus strains characterized in the study are shown in Table 2. For the cell tropism assessment of the HEV-D species, EV-94-E210 (characterized in I) and the prototype strains EV-68-Fermon and EV-70-J670/71 obtained from the American Type Culture Collection (ATCC, Manassas, VA) were used.

4.3 Experimental and evolutionary analysis

The experimental and evolutionary analysis methods are summarised in Tables 3 and 4. The details are included in the Material and Methods sections of the respective original publications.

4.4 Dataset collection for evolutionary analysis

The reference sequences for evolutionary analysis were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) (Benson et al., 2012). For the recombination analysis of the HEV-C species (see Results section), the complete genome sequences of the HEV-C species obtained with the search terms 'complete genome', 'enterovirus', 'coxsackievirus', 'echovirus' and 'poliovirus' were included. Because the objective of the analysis was to detect inter-typic recombination events, the sequences with more than 90 % complete genome similarity to any other sequence were removed from the analysis to relieve computational demands. For the EV-97 (HEV-B species) recombination analysis, the complete genome sequences with close resemblance to the EV-97 strains were retrieved from GenBank using the BLAST search (Altschul et al., 1990, Johnson et al., 2008). For the HEV-C selection analyses, all of the available complete VP1 sequences of EV-96, EV-99, CVA-21 and CVA-24 were retrieved from GenBank. The strains with identical amino acid sequences were removed from the dataset.

Table 1. The cell lines and primary cells used

Cell line	Tissue origin	Culture medium	Article
RD	Human rhabdomyosarcoma	Eagle's MEM[1]	I, II, IV
Hela	Human cervix	Eagle's MEM[1]	I, II, III
CACO-2	Human colorectal adenocarcinoma	Eagle's MEM[1]	I
A549	Human lung carcinoma	Eagle's MEM[1]	I
Hep-2C	Human larynx epidermoid carcinoma	Eagle's MEM[1]	I
HUH-7	Human hepatocellular carcinoma	Eagle's MEM[1]	I
SK-N-SH	Human neuroblastoma	Eagle's MEM[1]	I
KG-1	Human leukocyte (granulocytic)	IMDM[2]	IV
HL-60	Human leukocyte (granulocytic)	RPMI[3] 1640	IV
THP-1	Human leukocyte (monocytic)	RPMI[3] 1640	IV
U-937	Human leukocyte (monocytic)	RPMI[3] 1640	IV
RC-2A	Human leukocyte (monocytic)	RPMI[3] 1640	IV
Jurkat	Human leukocyte (T-cell)	RPMI[3] 1640	IV
MOLT	Human leukocyte (T-cell)	RPMI[3] 1640	IV
Raji	Human leukocyte (B-cell)	RPMI[3] 1640	IV
Endothelial	Human primary endothelial cells	D- MEM[1]	IV
Pancreatic islet	Human primary pancreatic islets	Ham's F10	IV
GMK	Green monkey kidney	Eagle's MEM[1]	I, IV
Vero	Green monkey kidney	Eagle's MEM[1]	I
3T3	Mouse embryo fibroblast	Eagle's MEM[1]	I
BHK21	Hamster kidney	Eagle's MEM[1]	I
RK13	Rabbit kidney	Eagle's MEM[1]	I
MDCK	Canine kidney	Eagle's MEM[1]	I
MDBK	Bovine kidney	Eagle's MEM[1]	I
L20B	Mouse fibroblast expressing human PVR	Eagle's MEM[1]	I
CHO-HCAR	Chinese hamster ovary cells expressing human coxsackievirus and adenovirus receptor (HCAR)	Eagle's MEM[1]	I
M4	Mouse fibroblast expressing ICAM-1	Eagle's MEM[1]	I

[1] Minimum essential medium
[2] Iscove's modified dulbecco's medium
[3] Roswell Park Memorial Institute medium

Table 2. The virus strains characterized

Species	Type	Strain	Country	Symp-toms	Sample type	Sequence region	Arti-cle
HEV-A	EV-76	KAZ00-14550	Kazakh-stan	AFP[1]	Stool	5'UTR, VP1, partial 3D	II
HEV-A	EV-90	LVA02-10337	Latvia	Healthy	Stool	5'UTR, VP1, partial 3D	II
HEV-B	EV-97	FIN03-2875	Finland (India)	Healthy	Stool	Complete genome	II[2]
HEV-C	EV-96	SVK03-24	Slovak Republic	AFP[1]	Stool	5'UTR, VP1, partial 3D	II
		FIN04-7	Finland (Thailand)	Healthy	Stool	Complete genome	II, III
		FIN05-2	Finland (China)	Healthy	Stool	Complete genome	II, III
		FIN05-5	Finland (China)	Healthy	Stool	5'UTR, VP1, partial 3D	II
		FIN05-10	Finland (China)	Healthy	Stool	5'UTR, VP1, partial 3D	II
		FIN05-12	Finland (China)	Healthy	Stool	5'UTR, VP1, partial 3D	II
		FIN05-14	Finland (China)	Healthy	Stool	5'UTR, VP1, partial 3D	II
		FIN06-7	Finland (China)	Healthy	Stool	5'UTR, VP1, partial 3D	II
HEV-D	EV-68	37-99	France	NA	Stool	Complete genome	I
	EV-94	E210	Egypt		Sewage	Complete genome	I, IV
		E430	Egypt		Sewage	VP1, partial 3D	I
		E435	Egypt		Sewage	VP1, partial 3D	I
		E438	Egypt		Sewage	VP1, partial 3D	I
		19-04	DRC[3]	AFP[1]	Stool	Complete genome	I

[1] Acute flaccid paralysis
[2] Complete genome sequence unpublished
[3]The Democratic Republic of Congo

Table 3. Experimental methods used

Virological methods		**Article**
Virus isolation	from stool	I, II
	from sewage	I
Virus purification	Plaque purification	I, II, IV
	End-point titration	II, III
Virus culture		I, II, III, IV
Infectivity assay	$TCID_{50}$[1]	I, II, III, IV
Growth curve analysis	Infection + $TCID_{50}$	I, IV
Immunofluorescence	In-house enterovirus specific Ab[2] +	I, IV
detection of virus	Immunofluorescence microscopy	
Ab prevalences	Serum neutralization assay	I, IV
Acid sensitivity	Acid sensitivity assay	I
Receptor usage	CPE[3]-protection assay	I
Molecular biology methods		
RNA-extraction		I, II, III
Reverse transcription PCR		I, II, III
cDNA synthesis		I, II, III
PCR	Primer walking strategy	I, II, III
Sequencing	Sanger sequencing	I, II, III
PCR-product purification	Agarose gel extraction	I, II, III
Cellular biology methods		
Cell culture		I, II, III, IV
Primary cell isolation		IV
Cell viability assays	WST (tetrazolium salt)	IV
	Live-Dead (calcein / ethidium homodimer-1)	IV
	Insulin-DNA ratio	IV
Cell detection	Immunofluorescence microscopy	I, IV

[1]50 % Tissue culture infectious dose
[2]Antibody
[3]Cytopathic effect

Table 4. Sequence analysis methods used.

	Method	Reference	Software
BLAST	blastn	(Altschul et al., 1990)	NCBI web site[1]
Sequence assembly			ContigExpress, Vector NTI 10.1
Alignment	ClustalW	(Thompson et al., 1994)	ClustalX v 1.81 [2] MEGA 3.1-5.0[3]
Substitution model	p-distance		MEGA 3.1-5.0
	Tamura-Nei	(Tamura & Nei., 1993)	
	Generalised time reversible	(Tavaré., 1986)	Datamonkey[4]
Phylogenetic tree construction	Neighbor Joining	(Saitou & Nei., 1987)	MEGA 3.1-5.0
	Maximum likelihood	(Felsenstein., 1981)	Tree-Puzzle v 5.2[5]
Recombination	Similarity plot	(Lole et al., 1999)	Simplot 2.5[6]
	Bootscanning	(Salminen et al., 1995)	
Selection	SLAC	(Kosakovsky Pond & Frost., 2005)	Datamonkey
	FEL		
	REL		
	McDonald-Kreitman test	(McDonald & Kreitman., 1991)	DnaSP v.5.10[7]

References for the software used:
1 (Johnson et al., 2008)
2 (Thompson et al., 1997)
3 (Kumar et al., 2004, Tamura et al., 2007, Tamura et al., 2011)
4 (Delport et al., 2010)
5 (Schmidt et al., 2002)
6 (Lole et al., 1999)
7 (Librado & Rozas., 2009)

5 Results

5.1 Genetic characterisation of the untypable enterovirus strains (I, II & III)

Three new enterovirus types, designated as EV-94, EV-96 and EV-97, were characterised in this thesis. An enterovirus strain is considered to form a new type when the strain has < 70 % nucleotide (nt) sequence similarity and < 85 % amino acid (aa) sequence similarity in the VP1-coding region with the strains representing the known enterovirus types and the strains of the proposed new type form a monophyletic group in this region (Oberste et al., 1999, Oberste et al., 2000). Since the initial isolation, several strains have been characterised for all of the new enterovirus types in Finland and elsewhere.

EV-94 was concurrently isolated from sewage in Egypt (4 strains) and acute flaccid paralysis (AFP) patients in the Democratic Republic of the Congo (DRC) (2 strains). A BLAST search suggested that EV-94 was related to the strains of the HEV-D species. The VP1 nt and aa sequence similarities (Table 5) and the phylogenetic analysis of this region (Fig. 4) suggested that the EV-94 strains form a novel type in the HEV-D species.

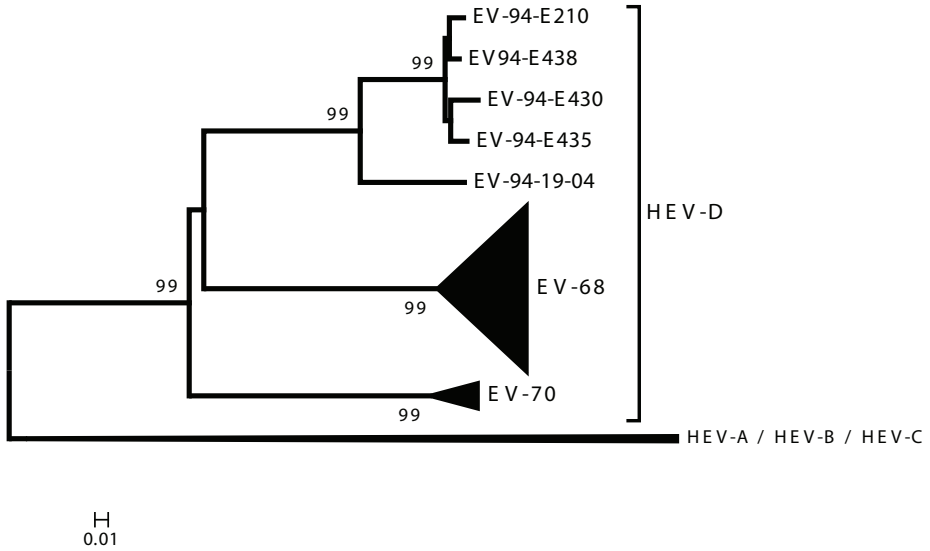


Figure 4. A phylogenetic tree constructed from the VP1-coding nt sequences of the HEV-D strains. The tree was constructed using the Neighbour-Joining method. The reliability of the inferred branching patterns was assessed using bootstrap test with 1000 replicates. The bootstrap values > 70 % are shown. The genetic distances were computed using the Tamura-Nei model for nt substitutions.

The first EV-96 strain (EV-96-SVK03-24) was isolated from a faecal sample of a four year old child with AFP in the Slovak Republic. Subsequently, similar viruses were detected from healthy children adopted from Southern Asia to Finland. The sequence similarities and phylogenetic analysis based on the VP1-coding region suggested that these virus strains form a new type in the HEV-C species (Fig. 5).

The EV-97 strain FIN03-2875 was isolated in Finland from a faecal sample from a healthy 3-year-old child adopted from India. In the phylogenetic analysis, this isolate grouped together with the HEV-B serotypes in the VP1 region but had only 68.4 % nt and 78.0 % aa sequence similarities with the closest enterovirus prototype strain (E-27). These results suggest that this virus strain represents a new enterovirus type (Fig. 6).

Currently, 20 strains of the EV-97 type have been detected, and complete VP1 sequences are known for 11 of the strains. In the phylogenetic analysis, the EV-97 strains grouped together with the E-27 strains. The pairwise nt and aa similarities between the EV-97 strains were 80.8-99.2 % and 92.0-99.3 %, respectively, and

the nt and aa similarities between the EV-97 and E-27 strains were 67.6-71.9 and 77.2-78.9 %, respectively. The VP1 phylogenetic tree showed a ladder-like structure, in which a strain isolated from a faecal sample from a 5-year-old boy with AFP in 1999 (Tao et al., 2010) formed an outgroup to all other strains. EV-97-FIN03-2875 clustered with two strains isolated from India and Pakistan.

Table 5. Nucleotide and amino acid sequence differences (%) between EV-94-E210 and other HEV-D strains

	Nucleotide sequence			Amino acid sequence		
	EV-94 19-04	**EV-70-J670/71**	**EV- 68-Fermon**	**EV94-19-04**	**EV 70-J670/71**	**EV 68-Fermon**
Complete genome	ND	75.8	73.7	97.0	86.9	84.8
5' UTR	ND	83.5	76.2			
VP 4	85.0	79.7	77.3	97.1	95.7	89.9
VP 2	86.0	74.8	74.2	98.8	84.4	84.7
VP 3	84.3	70.9	70.1	99.1	80.4	82.6
VP 1	86.3	67.8	67.7	97.1	73.6	71.4
2 A	84.1	73.6	69.6	93.9	85.2	82.3
2 B	81.5	75.1	76.4	92.9	94.9	85.9
2 C	83.5	77.8	77.4	96.1	90.0	88.2
3 A	86.1	78.3	78.3	96.6	92.1	89.9
3 B	86.4	66.7	75.8	100.0	95.5	86.4
3 C	84.5	76.3	72.9	96.7	88.0	88.0
3 D	89.9	82.7	77.0	97.8	95.2	90.6
3' UTR	ND	80.7	68.8			

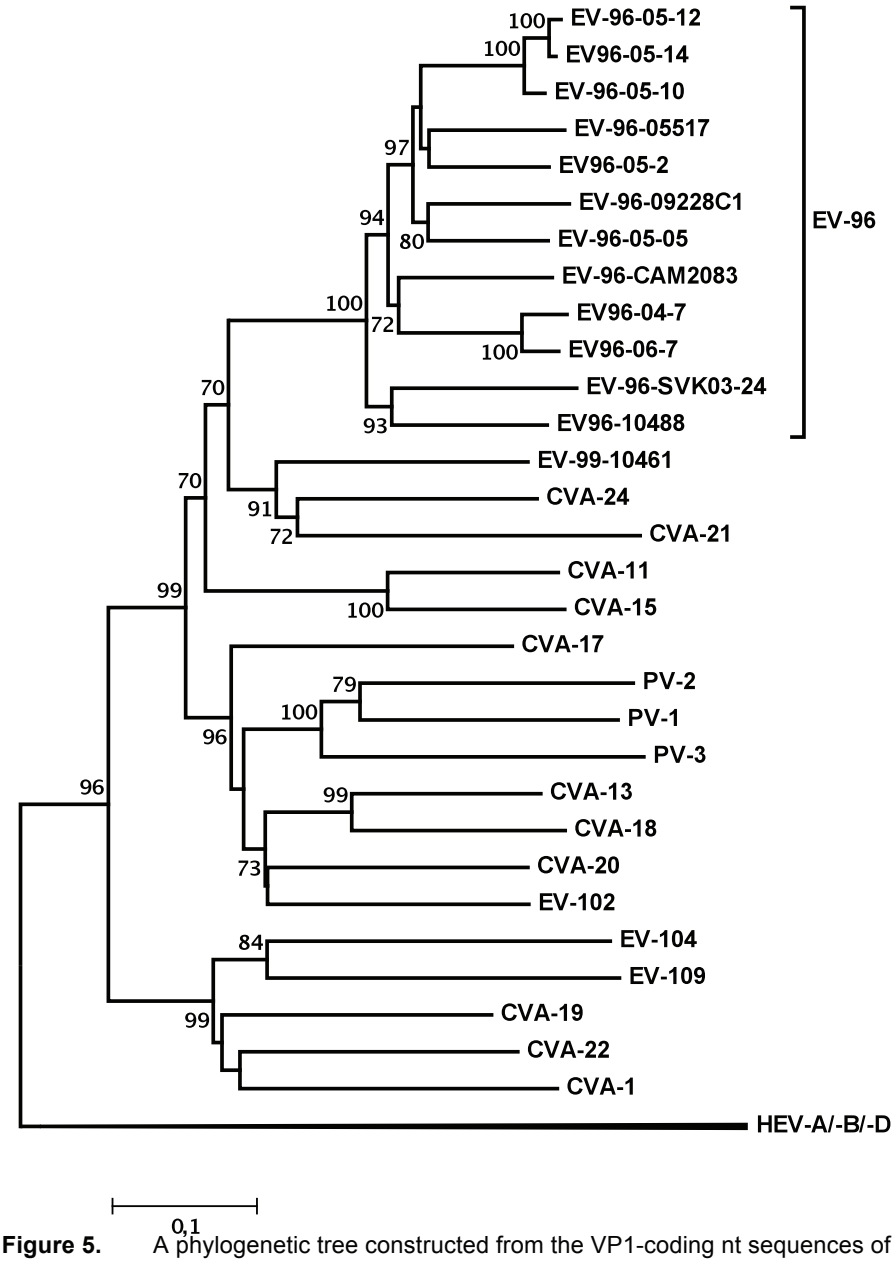


Figure 5. A phylogenetic tree constructed from the VP1-coding nt sequences of the EV-96 strains and the prototype strains of the other types of HEV-C species. The tree was constructed using the Neighbour-Joining method. The reliability of the inferred branching patterns was assessed using a bootstrap test with 1000 replicates. The bootstrap support values > 70 % are shown. The genetic distances were computed using the Tamura-Nei model for nt substitutions.

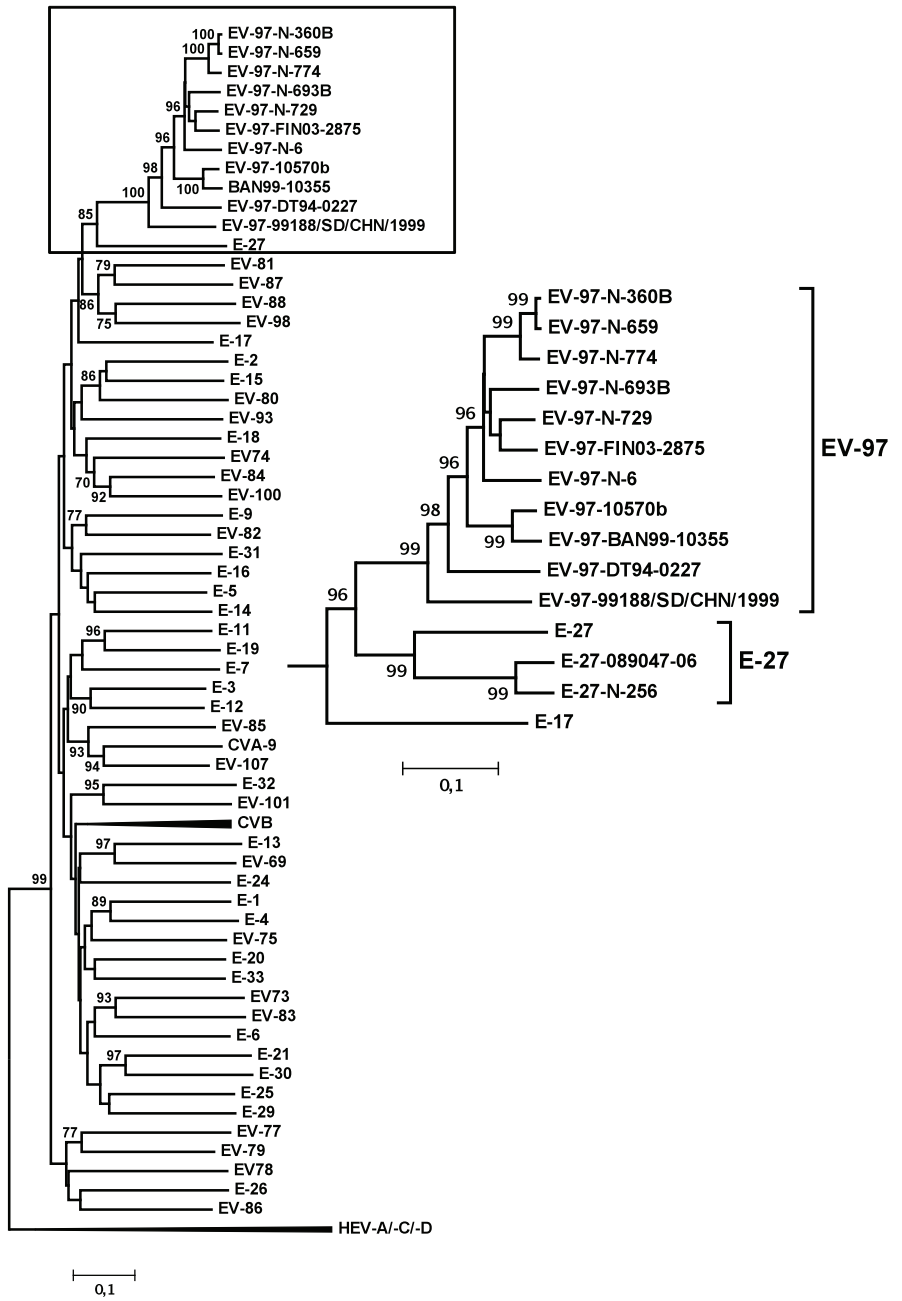


Figure 6. A phylogenetic tree constructed from the VP1-coding nt sequences of the EV-97 and E-27 strains and the prototype strains of HEV-B using the Neighbour-Joining method. The reliability of the tree topology was assessed using a bootstrap test with 1000 replicates. The bootstrap values > 70 % are shown. The genetic distances were computed using the Tamura-Nei model for nt substitutions.

5.2 Analysis of the complete genome sequences (I, III, V)

For further genetic characterisation and inference of evolutionary relationships and mechanisms, the complete genomes of two EV-94 strains, two EV-96 strains and one EV-97 strain were sequenced and analysed together with sequences derived from GenBank.

5.2.1 EV-94 in the HEV-D species (I)

Currently, the complete genome sequences are available for seven strains of the HEV-D species. High intra-typic similarities were detected between the two EV-94 strains and between the EV-68 strains in all regions of the genome (Table 5, Fig. 7). The phylogenetic trees constructed from the P1, P2 and P3 regions or the partial 3D sequences (more EV-94 strains) were essentially congruent (Fig. 8). Furthermore, the bootscanning analysis of the complete genome sequences indicated robust clustering of the EV-94 strains together in all regions of the genome (Fig. 7).

Between the EV-94 strains, approximately equal divergences were detected for the P1-, P2- and P3-coding regions of the genome in the nucleotide and amino acid sequence comparisons. Whereas for the EV-68 strains, nucleotide divergences were similar for the P1-, P2- and P3-coding regions, but the amino acid differences were slightly higher in the P1 region (Table 6). Between serotypes the distances were higher for the P1 than the P2 and P3 sequences. Of the distinct genes, the sequence divergences were highest in the VP1-coding region.

Table 6. Ranges of pairwise nucleotide and amino acid sequence divergences (%) in HEV-D P1-P3 regions for intratypic and intertypic comparisons

	Nucleotide sequence divergence (%)					
	P1		P2		P3	
	Intratypic	Intertypic	Intratypic	Intertypic	Intratypic	Intertypic
EV-94	14.4	28.4-29.1	16.5	22.6-24.5	11.9	19.7-23.9
EV-70	ND	28.4-32.0	ND	22.6-26.0	ND	19.7-24.6
EV-68	0.1-12.6	28.4-32.0	1.2-12.0	23.3-26.0	1.0-11.5	22.9-24.6
	Amino acid sequence divergence (%)					
EV-94	1.9	18.5-20.9	5.2	10.3-13.0	2.5	6.4-10.3
EV-70	ND	18.5-23.7	ND	10.3-15.2	ND	6.4-12.0
EV-68	0.3-6.4	19.6-23.7	0.5-2.5	11.8-15.2	0.4-2.8	9.7-12.0

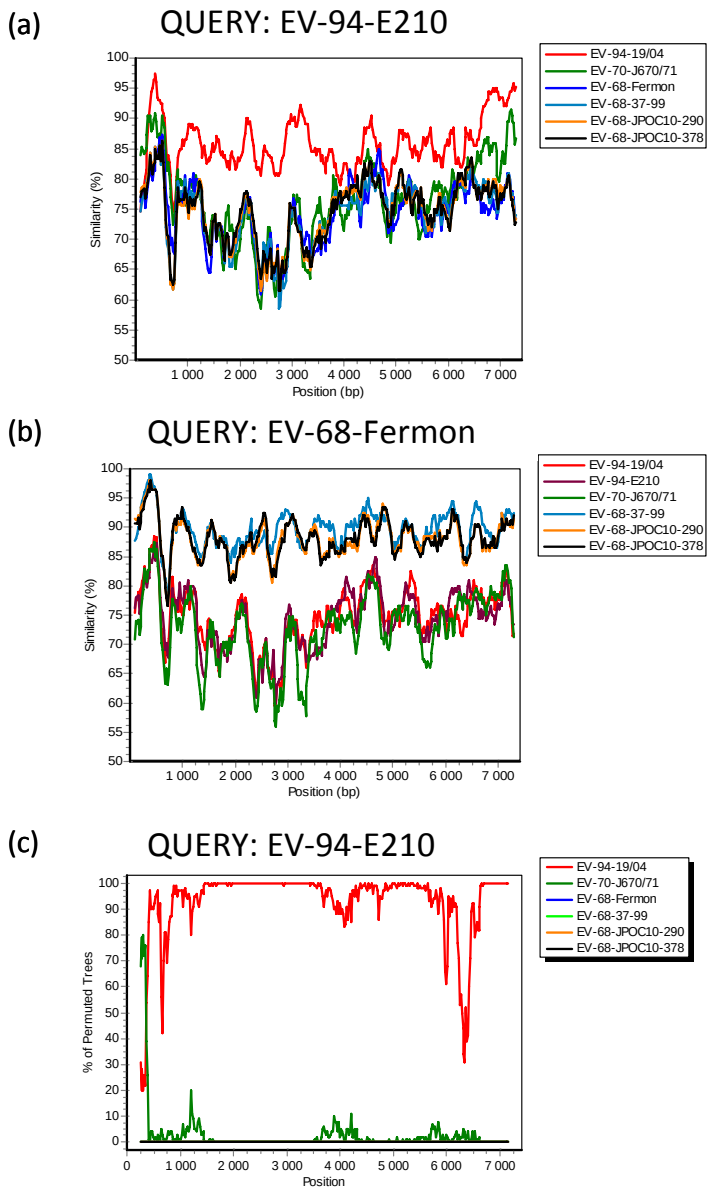


Figure 7. The similarity plots (a-b) and bootscanning analysis (c) of the complete HEV-D genomes. EV-94-E210 (a, c) and EV-68-Fermon (b) were used as query sequences. The similarity plot analysis was conducted using a sliding window of 200 nt moving in 20 nt steps. The bootscanning analysis was conducted using a sliding window of 500 nt moving in 20 nt steps. The genetic distances were computed using the Kimura 2-parameter model. The Neighbour joining method was used for the tree construction. The bootstrap values were calculated for 100 replicates. The transition to transversion ratio was estimated for each window.

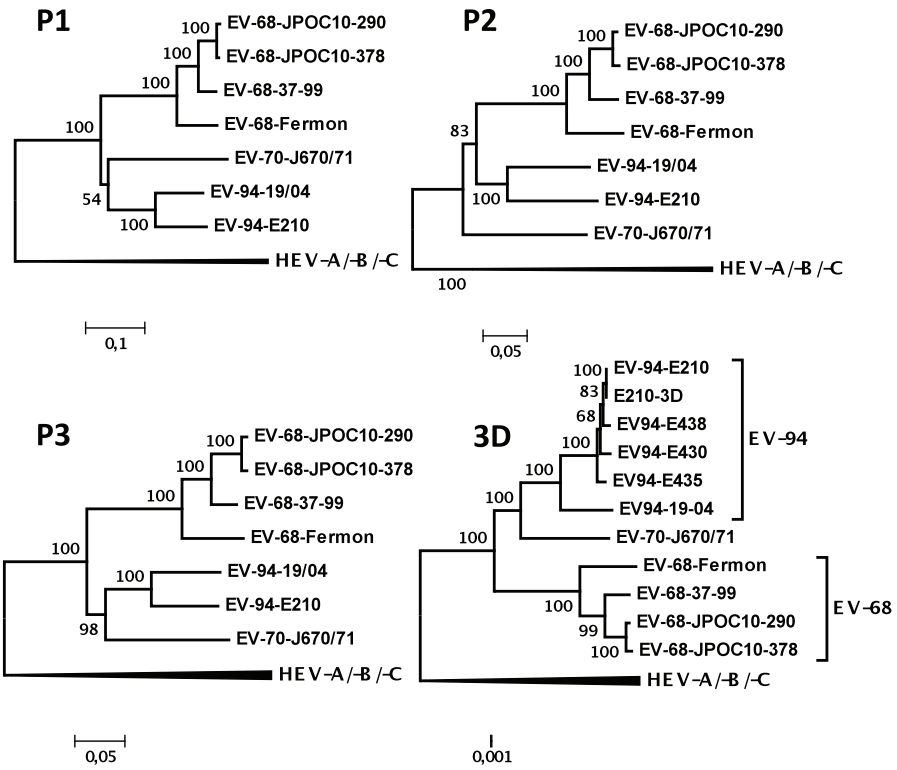


Figure 8. The phylogenetic trees constructed from the P1-, P2-, P3- and partial 3D-coding nucleotide sequences of the HEV-D strains. The trees were constructed using the Neighbour-Joining method. The bootstrap support values were calculated for 1000 replicates. The genetic distances were computed using the Tamura-Nei method.

5.2.2 EV-96 in the HEV-C species (II, V)

The complete genome sequences are known for five EV-96 strains. The similarity plot analysis suggested that the nt distances are approximately equal in the P1-, P2- and P3-coding regions between the strains FIN05-2, 05517 and 09228C1 (Fig. 9a). Similarly, the strains FIN04-7 and BAN00-10488 share equal similarities in all three regions (Fig. 9b). However, in the P2 and P3 regions the strains FIN04-7 and BAN00-10488 have low sequence similarity to the other three EV-96 strains.

The phylogenetic analysis of the EV-96 strains and the prototype strains of the other members of the HEV-C species suggested that the EV-96 strains are monophyletic only in the capsid-coding (P1) region (Fig. 1 in III). These results suggest that recombination has occurred between EV-96 and other HEV-C types. Recombination was further verified using similarity plot and bootscanning methods (Fig. 2 in III). The strains EV-96-FIN05-2, EV-96-05517 and EV-96-09228C1 grouped

together with CVA-24 and CVA-21 in the non-structural protein P2-coding region (Fig. 10b and Fig. 1b in III), whereas this clustering was not well-supported in the P3 coding region (Fig. 10c). In the P2 and P3 regions, the strains EV-96-FIN04-7 and EV-96-BAN00-10488 were clearly related to CVA-1, CVA-19, CVA-22, EV-104 and EV-109 prototype strains (Fig. 10 and Fig. 1c in III). Two apparent recombination sites were detected for the EV-96 strains FIN04-7 and FIN05-2 on the 5′ and 3′ ends of the capsid-coding region. The nt and aa sequence alignments suggested that the recombination sites might be within the 5'UTR-VP4 and at the 3′ end of the 2A protein coding regions.

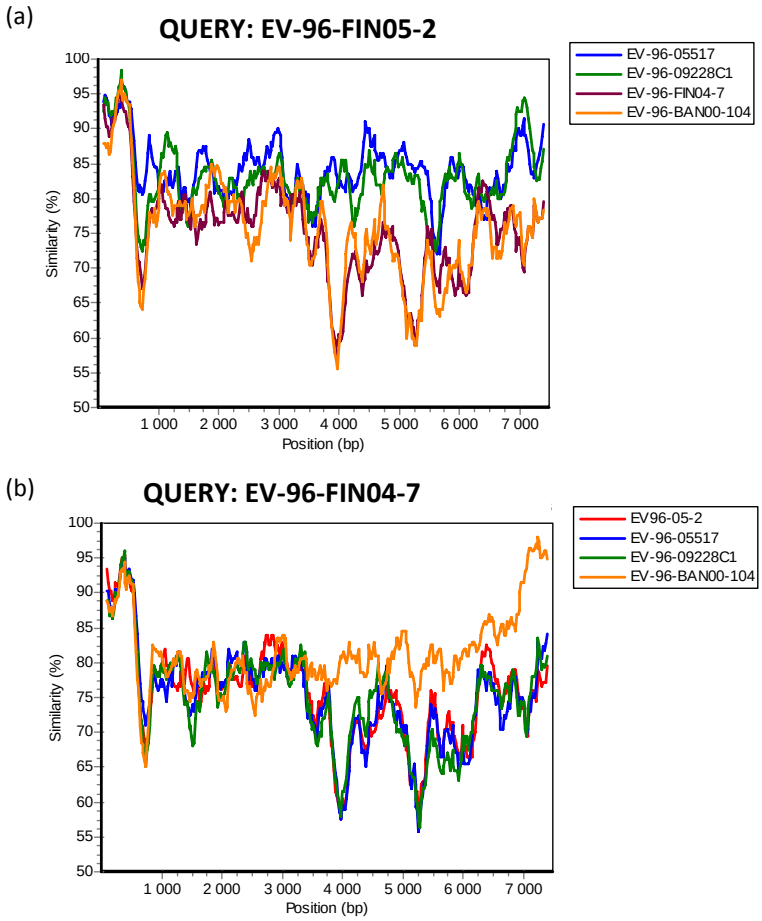


Figure 9. The similarity plots of complete EV-96 genomes using a sliding window of 200 nt moving in 20 nt steps. EV-96-FIN05-2 (a) and EV-96-FIN04-7 (b) were used as query sequences.

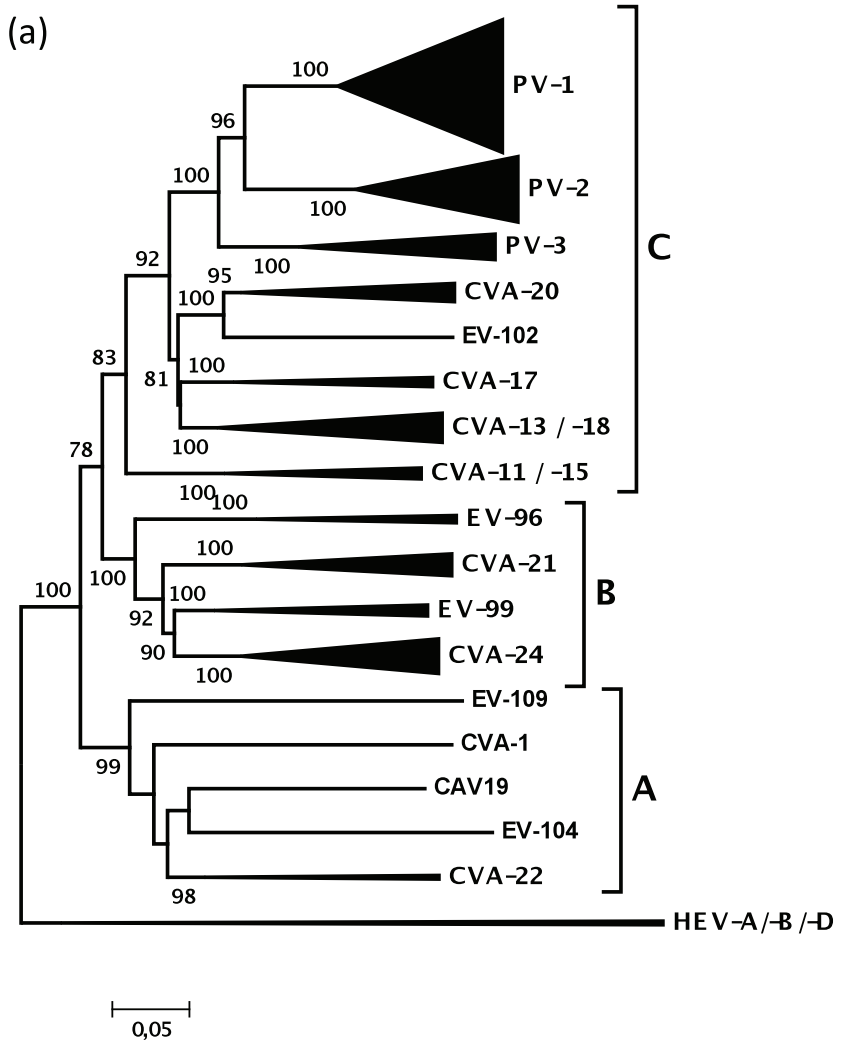
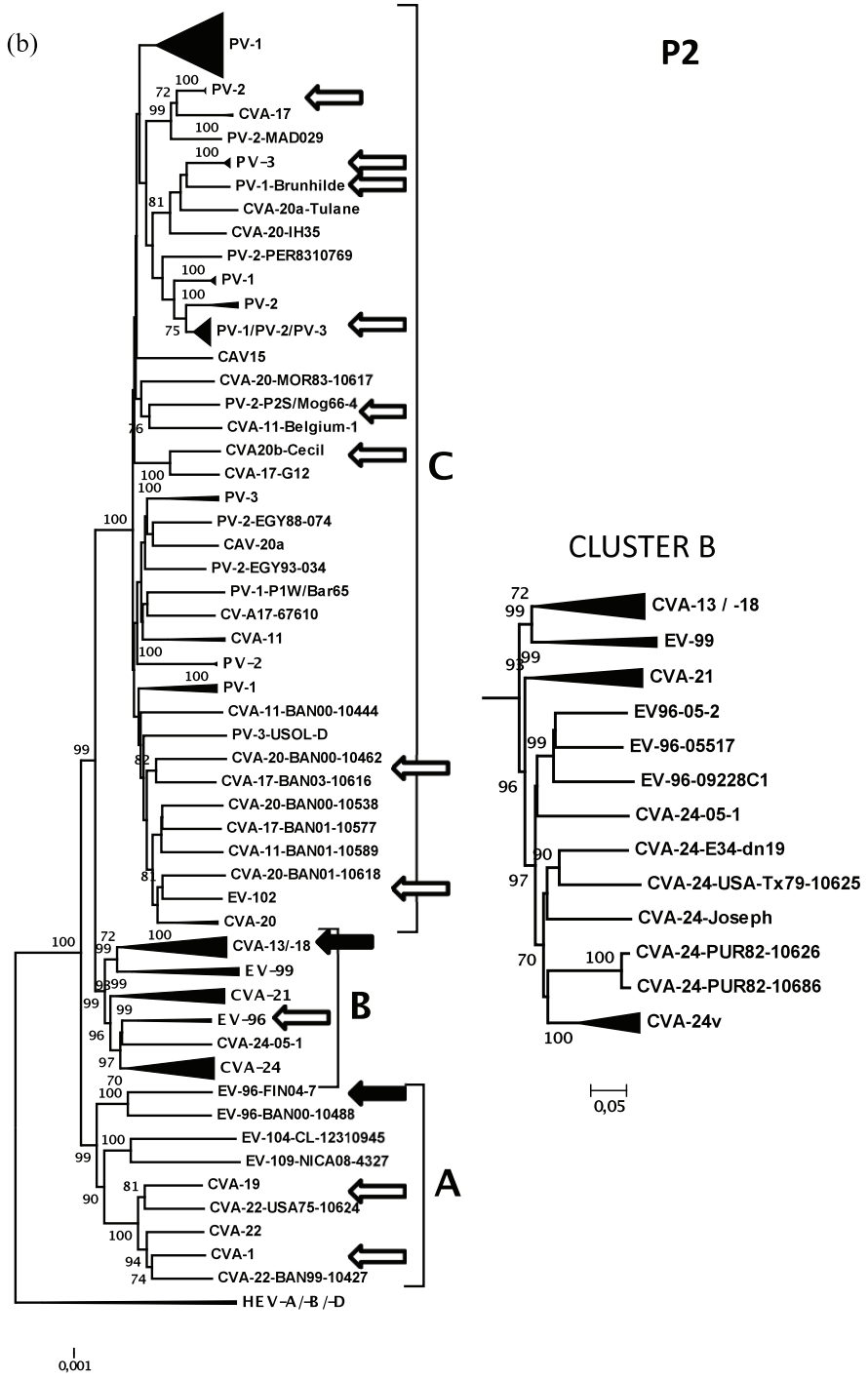
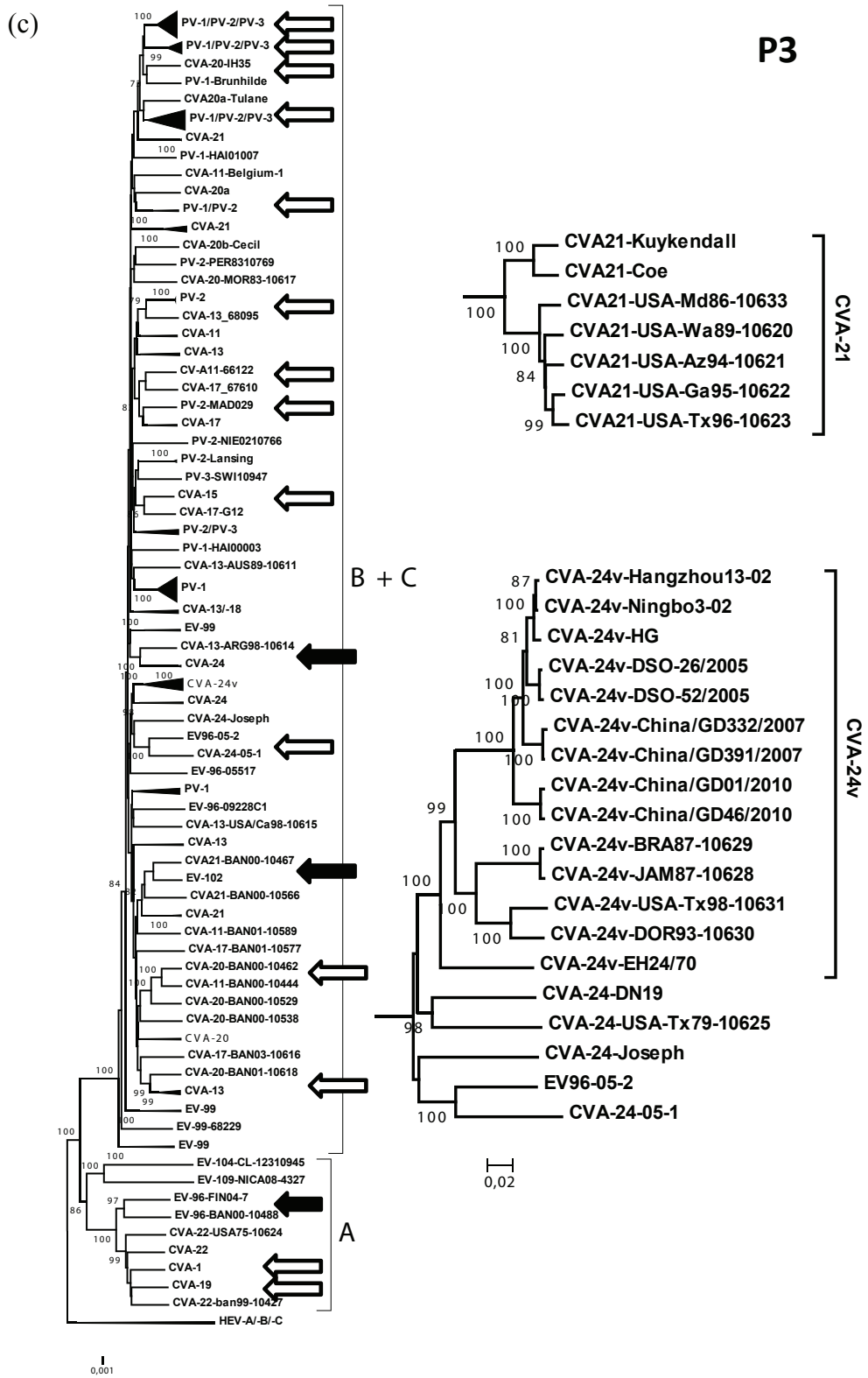


Figure 10. The phylogenetic trees constructed from the P1- (a), P2- (b) and P3- (c) coding nt sequences of the HEV-C strains. The strains that show robust (> 70% bootstrap support) incongruent clustering in comparison to the P1 clustering are indicated with arrows. The open arrows indicate recombination within the major clusters A, B or C, and the solid arrows indicate recombination between the major clusters. The trees were constructed using the Neighbour-Joining method. The bootstrap support values were calculated for 1000 replicates. The genetic distances were computed using the Tamura-Nei model for nt substitutions.


(b)
P2
PV-1
PV-2
CVA-17
PV-2-MAD029
PV-3
PV-1-Brunhilde
CVA-20a-Tulane
CVA-20-IH35
PV-2-PER8310769
PV-1
PV-2
PV-1/PV-2/PV-3
CAV15
CVA-20-MOR83-10617
PV-2-P2S/Mog66-4
CVA-11-Belgium-1
CVA20b-Cecil
CVA-17-G12
C
PV-3
PV-2-EGY88-074
CAV-20a
PV-2-EGY93-034
PV-1-P1W/Bar65
CV-A17-67610
CVA-11
PV-2
PV-1
CVA-11-BAN00-10444
PV-3-USOL-D
CVA-20-BAN00-10462
CVA-17-BAN03-10616
CVA-20-BAN00-10538
CVA-17-BAN01-10577
CVA-11-BAN01-10589
CVA-20-BAN01-10618
EV-102
CVA-20
CVA-13/-18
EV-99
CVA-21
EV-96
CVA-24-05-1
CVA-24
B
EV-96-FIN04-7
EV-96-BAN00-10488
EV-104-CL-12310945
EV-109-NICA08-4327
CVA-19
CVA-22-USA75-10624
CVA-22
CVA-1
CVA-22-BAN99-10427
A
HEV-A/-B/-D
0,001
CLUSTER B
CVA-13 / -18
EV-99
CVA-21
EV96-05-2
EV-96-05517
EV-96-09228C1
CVA-24-05-1
CVA-24-E34-dn19
CVA-24-USA-Tx79-10625
CVA-24-Joseph
CVA-24-PUR82-10626
CVA-24-PUR82-10686
CVA-24v
0,05


(c)
P3
PV-1/PV-2/PV-3
PV-1/PV-2/PV-3
CVA-20-IH35
PV-1-Brunhilde
CVA20a-Tulane
PV-1/PV-2/PV-3
CVA-21
PV-1-HAI01007
CVA-11-Belgium-1
CVA-20a
PV-1/PV-2
CVA-21
CVA-20b-Cecil
PV-2-PER8310769
CVA-20-MOR83-10617
PV-2
CVA-13_68095
CVA-11
CVA-13
CV-A11-66122
CVA-17_67610
PV-2-MAD029
CVA-17
PV-2-NIE0210766
PV-2-Lansing
PV-3-SWI10947
CVA-15
CVA-17-G12
PV-2/PV-3
PV-1-HAI00003
CVA-13-AUS89-10611
PV-1
CVA-13/-18
EV-99
CVA-13-ARG98-10614
CVA-24
CVA-24v
CVA-24
CVA-24-Joseph
EV96-05-2
CVA-24-05-1
EV-96-05517
PV-1
EV-96-09228C1
CVA-13-USA/Ca98-10615
CVA-13
CVA21-BAN00-10467
EV-102
CVA21-BAN00-10566
CVA-21
CVA-11-BAN01-10589
CVA-17-BAN01-10577
CVA-20-BAN00-10462
CVA-11-BAN00-10444
CVA-20-BAN00-10529
CVA-20-BAN00-10538
CVA-20
CVA-17-BAN03-10616
CVA-20-BAN01-10618
CVA-13
EV-99
EV-99-68229
EV-99
EV-104-CL-12310945
EV-109-NICA08-4327
EV-96-FIN04-7
EV-96-BAN00-10488
CVA-22-USA75-10624
CVA-22
CVA-1
CVA-19
CVA-22-ban99-10427
HEV-A/-B/-C
0,001
B + C
A
CVA21-Kuykendall
CVA21-Coe
CVA21-USA-Md86-10633
CVA21-USA-Wa89-10620
CVA21-USA-Az94-10621
CVA21-USA-Ga95-10622
CVA21-USA-Tx96-10623
CVA-21
CVA-24v-Hangzhou13-02
CVA-24v-Ningbo3-02
CVA-24v-HG
CVA-24v-DSO-26/2005
CVA-24v-DSO-52/2005
CVA-24v-China/GD332/2007
CVA-24v-China/GD391/2007
CVA-24v-China/GD01/2010
CVA-24v-China/GD46/2010
CVA-24v-BRA87-10629
CVA-24v-JAM87-10628
CVA-24v-USA-Tx98-10631
CVA-24v-DOR93-10630
CVA-24v-EH24/70
CVA-24-DN19
CVA-24-USA-Tx79-10625
CVA-24-Joseph
EV96-05-2
CVA-24-05-1
CVA-24v
0,02

To gain further insight into the recombination patterns within the HEV-C species, all of the HEV-C complete genome sequences available in GenBank (search 21.10.2011) were compared. At the P1 region, all of the strains clustered into three groups, which are designated as A (CVA-1, -19, -22, EV-104 and EV-109), B (EV-96, EV-99, CVA-21 and -24) and C (CVA-11/15, -13/18, -17, -20, EV-102, PV-1, -2 and -3) (Fig. 10a), with the branching order of cluster A diverging first from the common ancestor of clusters B and C. Within clusters B and C, the phylogenetic analysis suggested well-supported hierarchical branching orders. Within cluster B, EV-96 forms an outgroup to the CVA-21/CVA-24/EV-99 cluster, suggesting that EV-96 has diverged first from a common ancestor of group B. This divergence has been followed by the branching of CVA-21 from the ancestor of CVA-24/EV-99 and finally a splitting of the ancestor of CVA-24/EV-99 into two distinct types. Within cluster C, CVA-11/15 forms an outgroup to all of the other types, which is followed by a separation of the polioviruses from the CVA-13/18, CVA-17 and closely related EV-102/CVA-20 types. Within the poliovirus group, PV-3 appears to have diverged first from the common ancestor, followed by the divergence of PV-2 and PV-1.

At the P2 region, the three major clusters (A-C) remain congruent with two exceptions: two EV-96 strains group together with cluster A, and the CVA-13/18 strains group together with EV-99 in cluster B (Fig. 10b). However, within the major clusters, the P2 phylogenies are largely incongruent with those of the P1 region, suggesting promiscuous recombination within clusters A, B and C (in contrast to the apparently rare recombination between these clusters).

At the P3 region, all of the strains from clusters B and C merge into one large cluster (B+C), whereas cluster A remains separate from the others (Fig. 10c). Only a few P1 clusters remain congruent throughout the genome. Two notable exceptions are presented by some CVA-21 strains and CVA-24v (AHC-causing variant) strains, which show no traces of recombination.

Within cluster A, the EV-96 strains (FIN-04-7 and BAN00-10488) form a congruent subcluster throughout the P2 and P3 regions, and, likewise, EV-104 and EV-109 form a subcluster in these regions. However, at the P2 region, EV-104 and EV-109 are closer to CVA-1/-19/-22 than the EV-96 strains, whereas, at the P3 region, the EV-96 strains are closer to CVA-1/-19/-22 group. Furthermore, the similarity plot analysis suggests a very close relationship between the EV-96 strains and CVA-1/-19/-22 at the last ~500 nucleotides of the 3' end of the genome (Fig. 2a in III). These results suggest that multiple recombinations within cluster A have occurred during the evolutionary history of these strains.

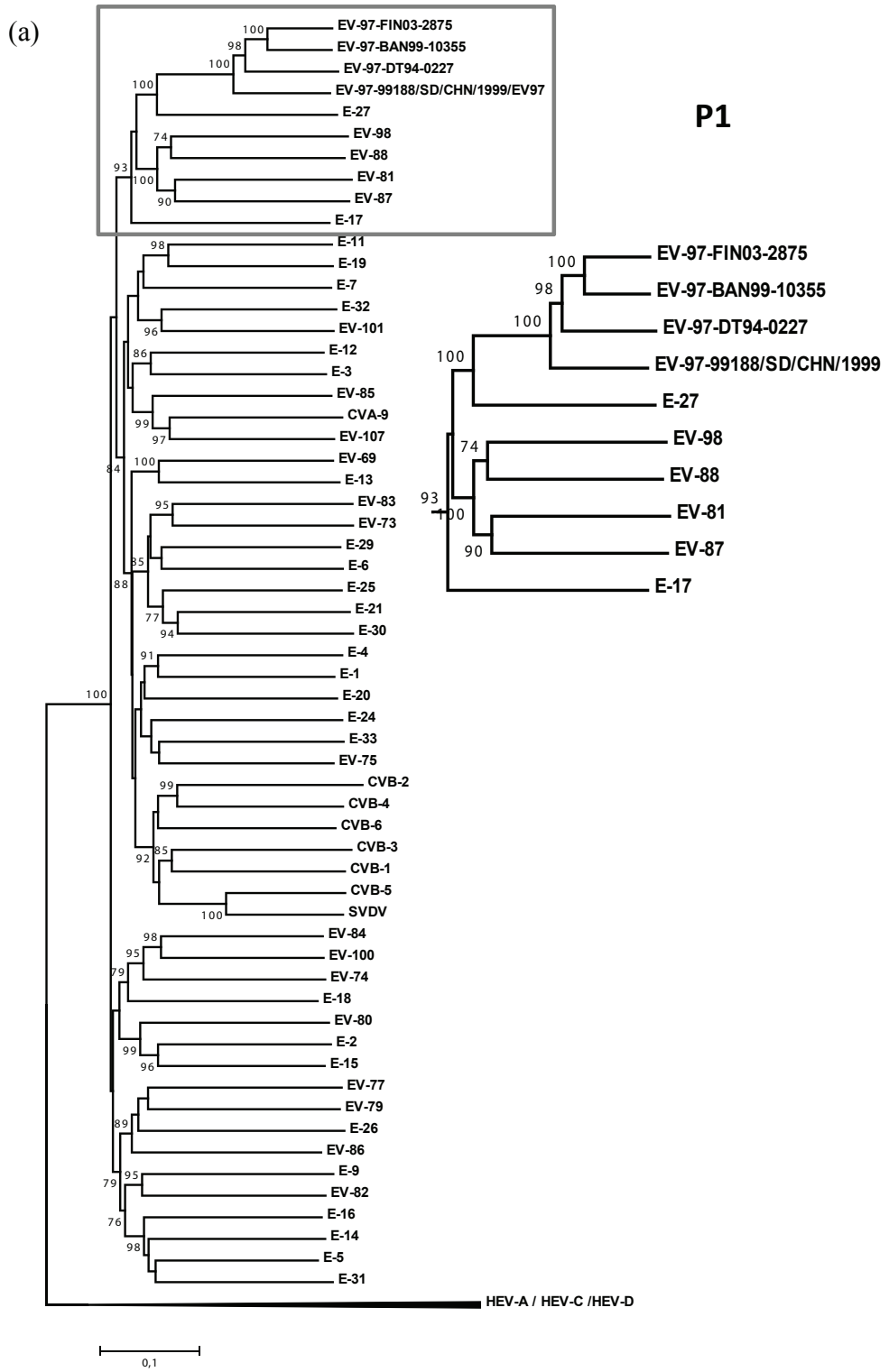
5.2.3 EV-97 in the HEV-B species (unpublished)

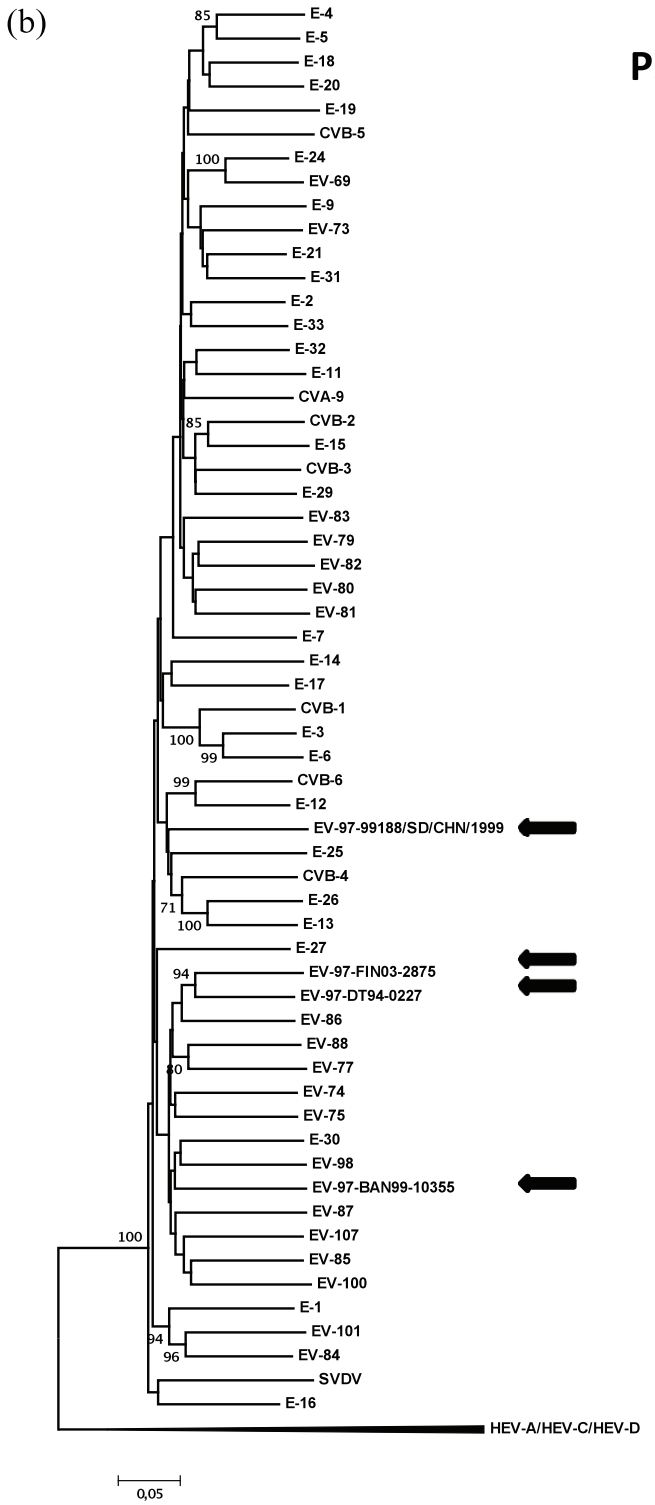
The complete genomes have been sequenced for four EV-97 strains. The phylogenetic analysis of EV-97 and the prototype strains of the HEV-B species showed

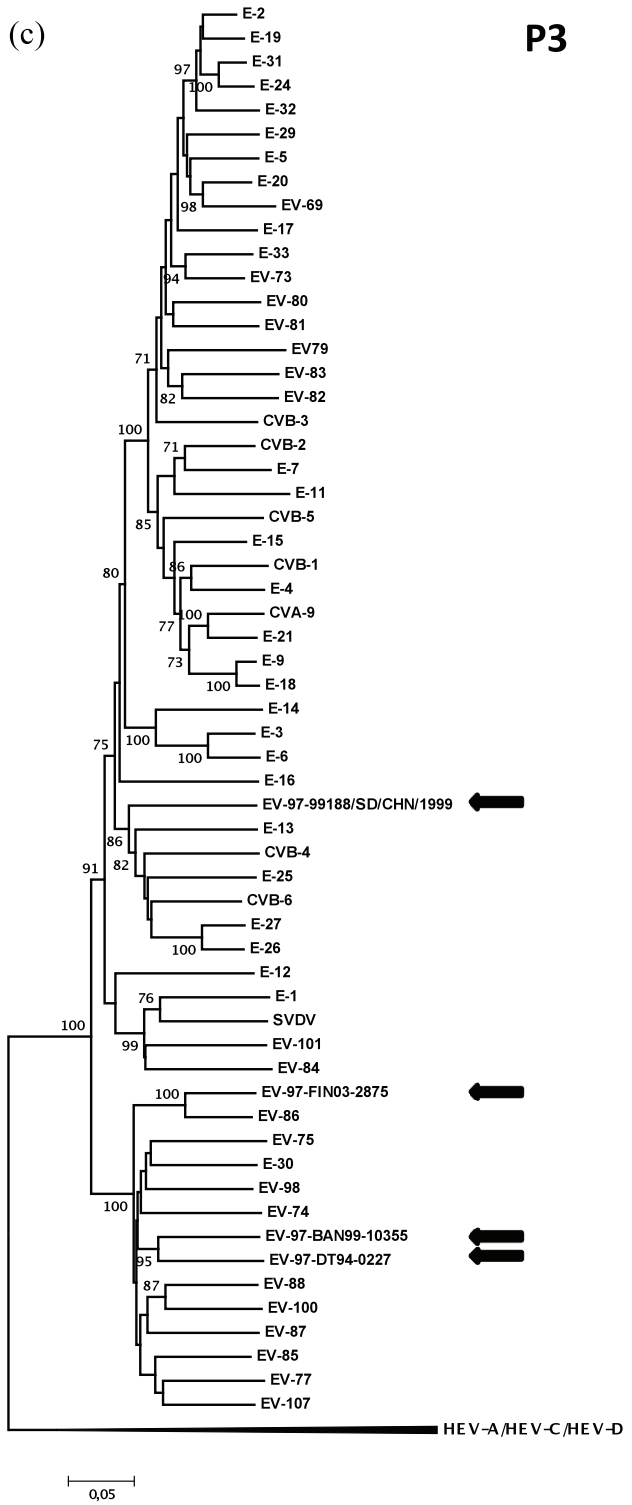
that the EV-97 strains formed a monophyletic group in the P1 region only (Fig. 11). In this region, the EV-97 strains and the closest type E-27 form a cluster with E-17 and the recently described HEV-B types EV-81, EV-87, EV-88 and EV-98 (Fig. 11a). In the P2 region, only a few of the prototype strains form well-supported clusters, and the clusters are almost unanimously incongruent to those observed at the P1 region. However, EV-97-FIN03-2875 and EV-97-DT94-0227 cluster together at P2 (Fig. 11b). In the P3 region, a more robust clustering pattern was observed (Fig. 11c). However, the P3-based phylogeny was largely incongruent to that of the P1 region. In the P3 region, the prototype strains formed two major clusters, the larger of which contained several subclusters. The strains EV-97-FIN03-2875, EV-97-BAN99-10355 and EV-97-DT94-0227 grouped together with the E-30, EV-74, EV-75, EV-85, EV-86, EV-87, EV-88, EV-98, EV-100 and EV-107 prototype strains. The strain EV-97-FIN03-2875 grouped together with EV-86, whereas the strains EV-97-BAN99-10355 and EV-97-DT94-0227 were phylogenetically close to each another.

A BLAST search using different genome regions of the EV-97 strains as query sequences was conducted to detect possible similarities between the circulating HEV-B strains and EV-97 strains in the non-structural protein-coding regions. The circulating strains with more than 83 % BLAST search similarity in the P3 region clustered together with EV-97-FIN03-2875, EV-97-BAN99-10355 and EV-97-DT94-0227 (Fig. 11d) and were included in the recombination analysis. At the P2 region, the EV-97 strains did not show bootstrap-supported clustering with the prototype strains, and circulating strains with high similarity were not detected with the BLAST search.

In the similarity plot analysis (Fig. 12a), no close relatives to EV-97-FIN03-2875 were found within the first 1000 nucleotides of the P2 region (corresponding to the nt sites 3300-4500 of the complete genome sequence). There was a sharp increase in the sequence similarities between EV-97-FIN03-2875 and EV-97-DT94-0227 corresponding to nt sites 4500-5200 of the complete genome sequence and an overlapping but shorter region of similarity between EV-97-FIN03-2875 and EV-86-BAN00-1035, CVB-3-MHC and E-30-TW/3182/01. High similarities with EV-97-FIN03-2875 were also detected for CVB-5-COXB5/Henan/2010 corresponding to nt sites 4800-5300 of the complete genome sequence and EV-100 corresponding to nt sites 5300-5800 of the complete genome sequence. At the 3' end of the P3 region that corresponds to nt sites 6000-7400, EV-97-FIN03-2875 and EV-86-BAN00-1035 showed high sequence similarities, whereas E-30-1167438/CBV-1-1167438 and E-7-LR11F7 showed high similarity to EV-97-FIN03-2875 at shorter regions near the 3' end of the genome. Bootscanning analysis (Fig. 12b) suggested phylogenetic clustering with the corresponding recombination partners.


(a)
EV-97-FIN03-2875
EV-97-BAN99-10355
EV-97-DT94-0227
EV-97-99188/SD/CHN/1999/EV97
E-27
EV-98
EV-88
EV-81
EV-87
E-17
P1
E-11
E-19
E-7
E-32
EV-101
E-12
E-3
EV-85
CVA-9
EV-107
EV-69
E-13
EV-83
EV-73
E-29
E-6
E-25
E-21
E-30
E-4
E-1
E-20
E-24
E-33
EV-75
CVB-2
CVB-4
CVB-6
CVB-3
CVB-1
CVB-5
SVDV
EV-84
EV-100
EV-74
E-18
EV-80
E-2
E-15
EV-77
EV-79
E-26
EV-86
E-9
EV-82
E-16
E-14
E-5
E-31
HEV-A / HEV-C /HEV-D
0,1


(b)
P2
E-4
E-5
E-18
E-20
E-19
CVB-5
E-24
EV-69
E-9
EV-73
E-21
E-31
E-2
E-33
E-32
E-11
CVA-9
CVB-2
E-15
CVB-3
E-29
EV-83
EV-79
EV-82
EV-80
EV-81
E-7
E-14
E-17
CVB-1
E-3
E-6
CVB-6
E-12
EV-97-99188/SD/CHN/1999
E-25
CVB-4
E-26
E-13
E-27
EV-97-FIN03-2875
EV-97-DT94-0227
EV-86
EV-88
EV-77
EV-74
EV-75
E-30
EV-98
EV-97-BAN99-10355
EV-87
EV-107
EV-85
EV-100
E-1
EV-101
EV-84
SVDV
E-16
HEV-A/HEV-C/HEV-D
0,05


(c)
P3
E-2
E-19
E-31
E-24
E-32
E-29
E-5
E-20
EV-69
E-17
E-33
EV-73
EV-80
EV-81
EV79
EV-83
EV-82
CVB-3
CVB-2
E-7
E-11
CVB-5
E-15
CVB-1
E-4
CVA-9
E-21
E-9
E-18
E-14
E-3
E-6
E-16
EV-97-99188/SD/CHN/1999
E-13
CVB-4
E-25
CVB-6
E-27
E-26
E-12
E-1
SVDV
EV-101
EV-84
EV-97-FIN03-2875
EV-86
EV-75
E-30
EV-98
EV-74
EV-97-BAN99-10355
EV-97-DT94-0227
EV-88
EV-100
EV-87
EV-85
EV-77
EV-107
HEV-A/HEV-C/HEV-D
0,05

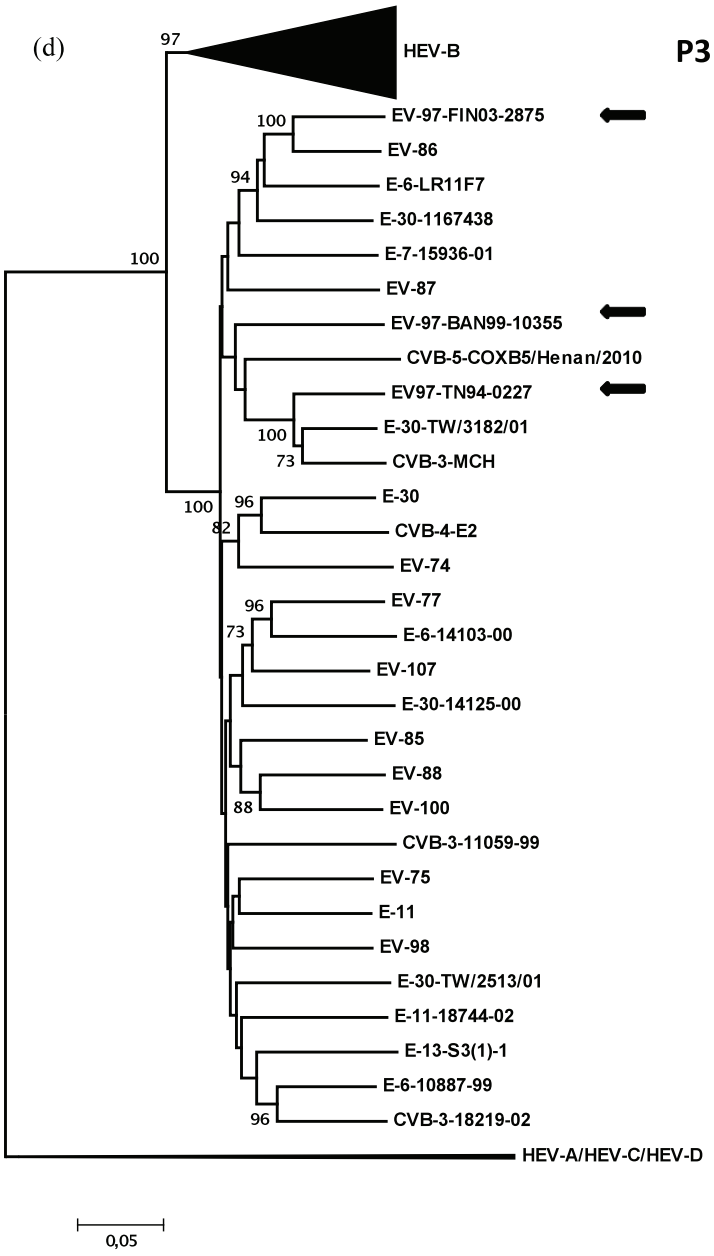


Figure 11. The phylogenetic trees constructed from the P1- (a), P2- (b) and P3- (c) coding nucleotide sequences of the HEV-B prototype strains and the P3 region of the HEV-B strains retrieved from GenBank (d). The arrows indicate EV-97 strains. The trees were constructed using the Neighbour-Joining method. The bootstrap support values were calculated for 1000 replicates. The genetic distances were computed using the Tamura-Nei model for nt substitutions.

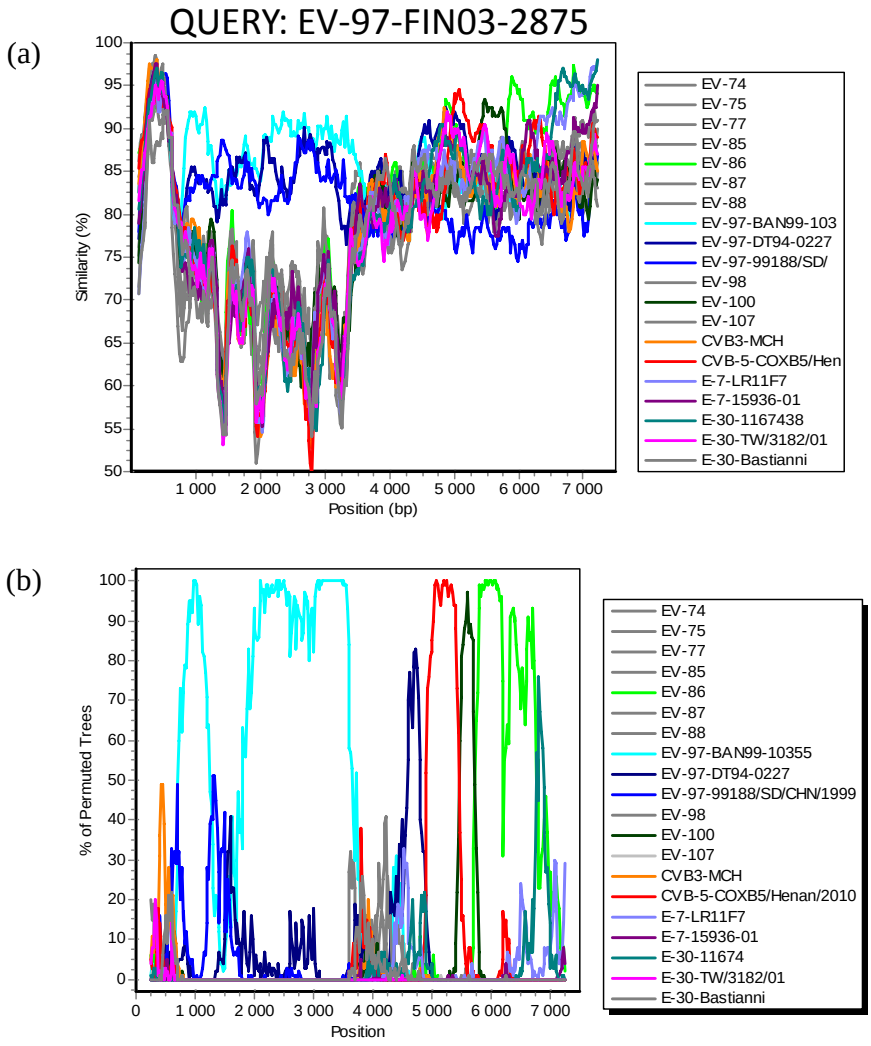


Figure 12. The similarity plot (a) and bootscanning (b) analysis of the HEV-B strains that cluster together with EV-97-FIN03-2875 in the P3 region (Fig. 11d). The similarity plot analysis was conducted using a sliding window of 200 nt moving in 20 nt steps with EV-97-FIN03-2875 as a query sequence. The bootscanning analysis was conducted using a sliding window of 500 nt moving in 20 nt steps. The genetic distances were computed with the Kimura 2-parameter nucleotide substitution model, and Neighbour-Joining method was used for the phylogenetic tree construction. The bootstrap values were calculated for 100 replicates. The transition to transversion ratio (Ts/Tv) was estimated for each window.

Out of the three EV-97 strains derived from GenBank, EV-97-DT94-0227 showed similarity to E-30-TW/3182/01 and CVB-3-MCH at the 3' end of the genome (5900-7400), whereas EV-97-99188/SD/CHN/1999 showed larger divergences at the non-structural region. However, E-9-DM and E-30/Zhejiang/17/03/CSF show the closest relation to this strain throughout the P2 and P3 regions (4000-7400). Likewise, the similarities between EV-97-BAN99-10355 and the other HEV-B strains were less than 90 % throughout the non-structural region.

5.3 Phylogenetic analysis of the 5'UTR (II)

The complete genome sequence analyses presented here and in other studies (see Chapter 6.3) suggest promiscuous recombination among the HEV-A, HEV-B and HEV-C strains. At the protein-coding regions, recombination appears to occur exclusively between the strains of the same species.

In contrast to the coding regions of the enterovirus genome, where all of the enterovirus strains cluster into four groups (representing the species HEV-A to –D), only three clusters were observed in the trees constructed from the 5'UTR sequences (Fig. 13). Cluster I is composed of strains from the HEV-C and HEV-D species, cluster II is composed of strains from the HEV-A and HEV-B species (Hyypia et al., 1997), and cluster III is composed of the HEV-C types EV-104 and EV-109 (Tapparel et al., 2009, Yozwiak et al., 2010).

The incongruity between the clustering patterns of the 5'UTR and other regions of the genome suggests that at least two ancient inter-species recombination events and several inter-typic recombination events have occurred within the 5'UTR during the evolutionary history of the enteroviruses (see Chapter 6.2.2.4). In this work (II), the recently discovered HEV-A types EV-90 and EV-91 were shown to cluster together with types from the HEV-C and HEV-D species (Fig. 13b). This clustering strongly suggests that an additional inter-species recombination event occurred between the ancestor of EV-90/91 and a member of the 5'UTR cluster I (i.e., a strain belonging to the HEV-C or HEV-D species) or between EV-90 or EV-91 and a member of the 5'UTR cluster I followed by an intertypic recombination event between EV-90 and EV-91. Two other recently described HEV-A types, EV-76 and EV-89, form an outgroup to the 5'UTR cluster II (strains of the HEV-A and HEV-B species), whereas EV-68 forms an outgroup to the 5'UTR cluster I (strains of HEV-C and HEV-D species).

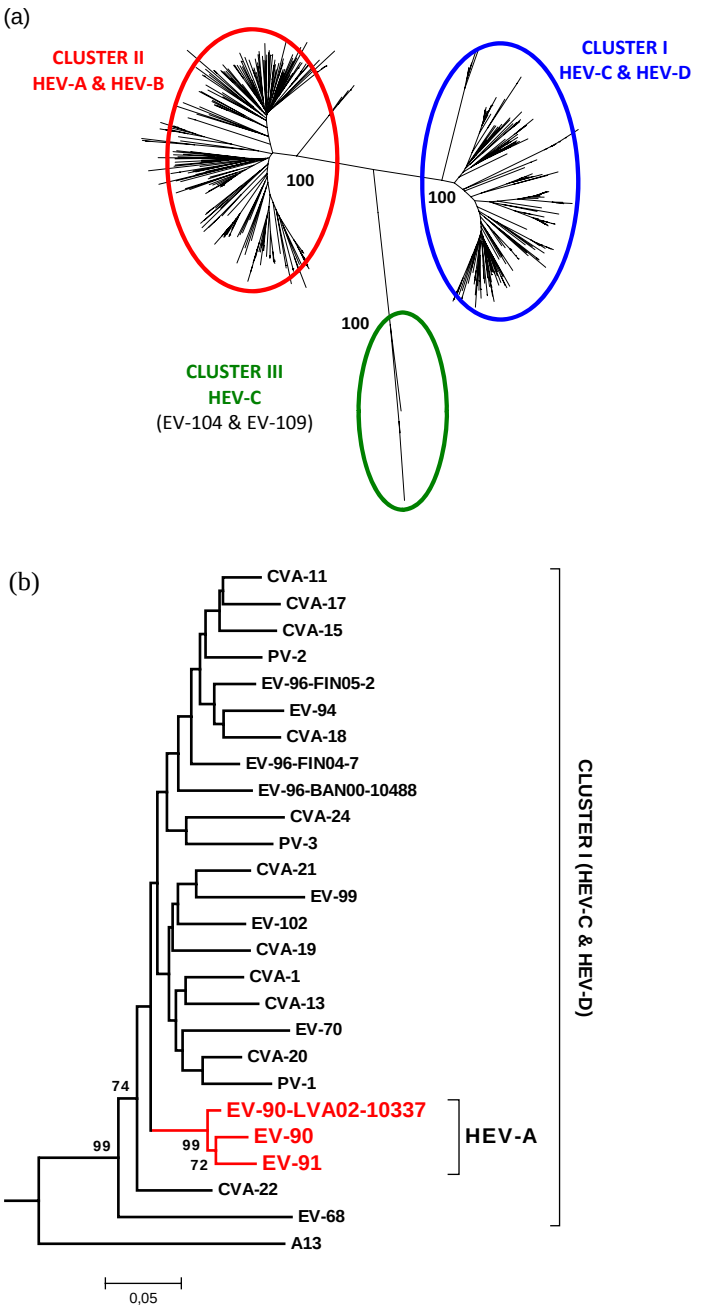


Figure 13. The phylogenetic trees constructed from the 5'UTRs of the strains representing all of the HEV species (a) and the prototype strains forming cluster I (b). The trees were constructed using the Neighbour-Joining method. The bootstrap support values were calculated for 1000 replicates. The genetic distances were computed using the Tamura-Nei model. To exclude the possible recombination site, only the first 500 nts of the 5'UTR were used. The bootstrap support values are shown only for major clusters (a) or clusters with a value > 70 (b).

5.4 Evolutionary patterns of the VP1-coding region: EV-96 as a model type (II, III, V)

To elucidate mechanisms by which new enterovirus types have originated and evolved, the mutation patterns in the VP1 region of EV-96 were studied and compared to closely related EV types. EV-96 was chosen for this analysis, because the most sequence data have accumulated from this type compared to the other new EV-types described in this thesis. As discussed above, due to frequent recombination, different regions of the enterovirus genome can be considered to evolve semi-independently. Because recombination in the capsid-coding region is rare, and molecular typing efforts have provided abundant sequence data for the VP1 region, the mutation patterns between virus lineages can be reliably assessed using this region.

5.4.1 Sequence diversity and phylogenetic analysis of EV-96 (I)

A wide variation in the nucleotide and amino acid sequences of the VP1-coding region was observed among the EV-96 strains (Table 7). The divergences were as high as 75 % at the nt sequence level and 89 % at the aa sequence level, which corresponded to 229 (of 927) nt and 33 (of 309) aa substitutions among the EV-96 strains.

Table 7. The pairwise nucleotide (lower left) and amino acid (upper right) similarities between the EV-96 strains in the VP1-coding region (%). The similarities within the intra-typic subclusters are indicated with colours: orange (A), green (B1), blue (B2).

	EV-96-05-12	EV96-05-14	EV-96-05-10	EV-96-05517	EV96-05-2	EV-96-09228C1	EV-96-05-05	EV-96-CAM2083	EV96-04-7	EV96-06-7	EV-96-SVK03-24	EV96-10488
EV-96-05-12		98.1	95.8	93.9	94.5	93.5	93.5	92.9	91.9	92.2	89.3	91.6
EV96-05-14	98.7		96.4	95.1	95.1	93.9	94.5	94.2	92.2	93.2	90.6	92.9
EV-96-05-10	96.3	96.3		95.1	95.1	95.5	94.5	93.9	92.6	92.6	91.6	92.2
EV-96-05517	83.8	84.0	83.9		95.5	94.2	93.5	94.2	92.6	92.6	90.0	92.6
EV96-05-2	84.1	84.4	84.8	84.8		93.9	94.8	94.2	93.5	92.9	91.3	92.6
EV-96-09228C1	82.4	82.7	83.3	81.7	83.2		94.2	93.2	91.6	91.6	90.3	91.9
EV-96-05-05	83.1	83.6	84.1	83.4	84.0	84.4		92.6	91.6	91.9	90.9	91.9
EV-96-CAM2083	79.8	80.4	80.9	80.8	81.9	79.8	80.3		92.9	94.2	90.9	93.9
EV96-04-7	80.4	80.8	81.2	78.6	79.9	79.1	80.7	81.2		97.1	89.3	90.9
EV96-06-7	80.6	81.4	80.6	79.6	79.6	79.6	80.6	81.1	94.6		90.3	92.2
EV-96-SVK03-24	76.1	76.3	76.6	76.9	78.3	77.2	77.3	77.9	75.3	76.9		92.2
EV96-10488	79.1	79.4	79.9	78.6	79.3	78.4	78.1	78.4	78.6	78.2	80.4	

In the phylogenetic tree, the EV-96 strains form two major clusters (Fig. 14), which are designated as A and B. The nucleotide sequence similarities within the subclusters were 80.4 % and 78.6-96.3 % (mean 83.6 %; S.E. 0.7) for clusters A and B, respectively, and the mean sequence similarity between the subclusters was 77.8 % (S.E. 0.9) ranging from 75.3 % to 79.9 %.

The corresponding amino acid sequence similarities within the cluster were 92.2 % and 91.6-98.1 % (mean 93.9 %; S.E. 0.9) for clusters A and B, respectively. Between the subclusters, the aa sequence similarities ranged from 89.3 % to 93.9 % (mean 91.3 % S.E. 1.2). The phylogenetic analysis based on the amino acid sequences did not support intra-typic subclustering, which was observed with the nucleotide sequences in the VP1 region.

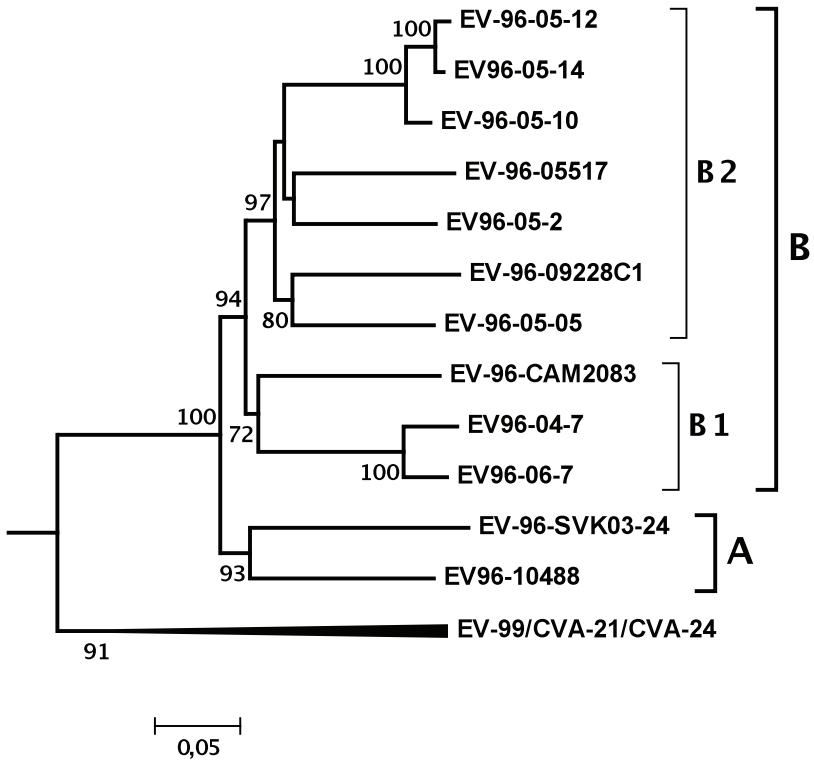


Figure 14. The phylogenetic relationship of the EV-96 strains. The tree was constructed from the VP1-coding sequence using the Tamura-Nei model of substitution and the Neighbour-Joining method of tree construction. The bootstrap support values were calculated for 1000 replicates.

5.4.2 Amino acid substitution pattern (V)

In the VP1 region, 242 out of 309 amino acid sites (78 %) were conserved among all of the EV-96 strains (Fig. 15). At 27 of 67 variable sites, mutations unique to single strains were observed. In contrast, 14 (4.5 %) amino acid sites showed high polymorphism with more than two different amino acid residues observed among the strains.

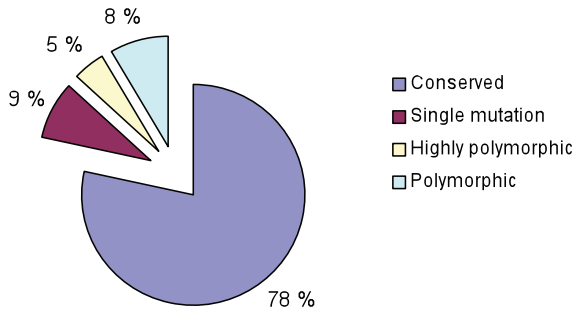
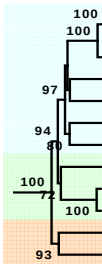


Figure 15. The proportions of conserved and polymorphic amino acid sites among the EV-96 strains in the VP1 region. At single mutation sites, amino acid substitution was only detected in a single strain, whereas, at highly polymorphic sites, more than two different amino acids were detected among the strains.

Only a few of the variable sites corresponded to the subclustering of the EV-96 nt phylogeny, i.e., no cluster-specific amino acid signature was observed between clusters A and B. The sole exception was site 27 (leucine L vs. isoleucine I). At some sites, a distinct amino acid mutation had independently occurred several times (as judged by clustering at the nt level) (Fig. 16). For example, at site 91, the majority of the strains contain isoleucine, whereas three divergent strains contain valin (V).

The biochemical properties of amino acid side chains (e.g., size, shape, charge, hydrogen-bonding capacity and chemical reactivity) determine the folding of a protein. Therefore, the aa substitutions that change a property e.g., hydrophobicity of a given site are more likely to change the structure of the virus capsid. Altogether, 31 amino acid sites showed substitutions that changed biochemical properties of the corresponding aa residues. Of these, 13 sites were mutations observed in single strains only. None of the mutations were specific for any of the intratypic clusters of EV-96.



	Cluster	5	42	91	94	102	103	117	174	225	227	228	230	233	265	295	298
EV-96-05-12	B2	L	S	I	M	V	E	N	N	D	T	T	S	**T**	V	N	**A**
EV96-05-14	B2	L	S	I	**I**	V	E	N	N	D	T	T	S	**T**	V	N	T
EV-96-05-10	B2	L	S	**V**	M	T	**D**	**S**	N	**G**	T	T	S	**T**	V	**G**	**A**
EV-96-05517	B2	L	S	I	**I**	T	E	N	**S**	D	T	**A**	**A**	**T**	V	**G**	**A**
EV96-05-2	B2	L	S	I	M	T	E	N	**S**	D	**S**	T	S	**T**	**I**	N	**A**
EV-96-09228C1	B2	L	S	**V**	M	**I**	E	**S**	N	**G**	T	**A**	G	A	V	N	**A**
EV-96-05-05	B2	**I**	S	**V**	M	T	E	N	N	D	T	T	S	**T**	V	N	T
EV-96-CAM2083	B1	L	**T**	I	M	**I**	E	N	**S**	D	A	I	S	A	V	**G**	T
EV96-04-7B	B1	L	S	I	M	T	E	N	T	D	**S**	T	N	**T**	V	D	**A**
EV96-06-7A	B1	L	S	I	M	T	E	N	T	D	N	T	S	A	V	D	T
EV-96-SVK03-24	A	**I**	S	**V**	M	T	**D**	N	N	D	D	T	S	A	**I**	S	T
EV96-10488	A	**I**	**T**	I	M	T	A	N	N	D	D	**A**	**A**	A	V	N	T

Figure 16. The VP1 aa sites of EV-96 strains that show parallel evolution. The EV-96 subclusters are indicated with colours: orange (A), green (B1), blue (B2).

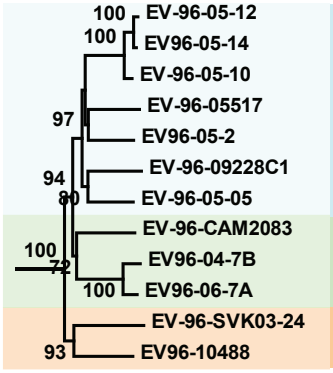
5.4.3 Codon-specific selection in the EV-96 VP-1 coding region (V)

Codon-specific non-synonymous to synonymous substitution frequency ratios (dN/dS or Ka/Ks) were assessed using MEGA5 (via HyPhy) and single likelihood ancestor counting (SLAC), fixed effects likelihood (FEL) and random effects likelihood (REL) methods available at the Datamonkey facility (www.datamonkey.org) to detect signatures of selection operating on the VP1 protein-encoding gene of EV-96. Because synonymous (silent) mutations are apparently neutral (free from natural selection), unless there is codon usage bias, the low dN/dS suggests negative (purifying) selection, and the high dN/dS suggests positive selection for a given codon. Under neutral evolution the rates of non-synonymous and synonymous substitutions are expected to be equal. For this analysis, the phylogenetic relationships between the EV-96 VP1 gene sequences were inferred with the general time reversible (GTR) substitution model and the phylogenetic trees were reconstructed by the Neighbour-Joining method.

This analysis suggested that strong negative selection was occurring over most of the codons of the EV-96 VP1-coding region. However, elevated dN to dS ratios were detected for some codons by all of the methods, and a subset of methods suggested that positive selection was occurring for amino acids at sites 104, 105, 227, 230 and 295 (Table 8). At these sites, there were strong amino acid polymorphism within and between subclusters (Fig. 17).

Table 8. The EV-96 codons with elevated dN/dS. The statistically significant evidence of positive selection is shown in boldface. The significance level (p-value/Bayes Factor/posterior probability) of 0.1 or Bayes factor of 50 (in REL analysis) was used as a confidence limit. Altogether, five positively selected and 295 negatively selected sites were detected with at least one method.

	Normalized dN-dS				Amino acid composition
Codon	HyPhy	SLAC	FEL	REL	(n of strains)
104	1.158	0.863	**2.514**	**0.182**	A (3), L (1), S (1), M (4), V (1), T(2)
105	2.110	1.747	**3.378**	**0.253**	I (1), A (2), V (1), S (5), T (3)
227	0.064	0.116	-0.723	**0.065**	T (6), D (2), A (1), S (2), N (1)
230	1.309	1.044	0.616	**0.966**	G (1), A (2), S (8), N (1)
295	-1.680	-1.410	-2.272	**0.990**	N (6), G (3), S (1)



Strain	Cluster	104	105	227	230	295
EV-96-05-12	**B1**	M	S	T	S	N
EV96-05-14	**B1**	M	S	T	S	N
EV-96-05-10	**B1**	V	S	T	S	G
EV-96-05517	**B1**	L	A	T	A	G
EV96-05-2	**B1**	T	T	S	S	N
EV-96-09228C1	**B1**	A	I	T	G	N
EV-96-05-05	**B1**	T	T	T	S	N
EV-96-CAM2083	**B2**	S	S	A	S	G
EV96-04-7	**B2**	A	T	S	N	D
EV96-06-7	**B2**	M	A	N	S	D
EV-96-SVK03-24	**A**	A	V	D	S	S
EV96-10488	**A**	M	S	D	A	N

Figure 17. The amino acid sites in the VP1 protein that show evidence of positive selection.

5.4.4 Amino acid substitution patterns: a comparison between types (V)

To compare the evolutionary patterns between enterovirus types and gain insight into the origin of new enterovirus types, the VP1 sequences of the EV-96 strains were compared to those of their closest relatives, CVA-21, EV-99 and CVA-24. In the capsid-coding P1 region these viruses form a subcluster B within the HEV-C species (Fig. 10a) and show a hierarchical branching order that corresponds to serotype specificity (see above) (Jiang et al., 2007, III).

The serotypes had within-type minimum amino acid sequence similarities of 89.3% for EV-96, 90.9 % for CVA-21, 87.7 % for EV-99 and 88.2 % for CVA-24. The sequence similarities between types are shown in Table 9.

Table 9. The ranges of pairwise nucleotide (lower left) and amino acid (upper right) similarities between the EV-96, EV-99, CVA-21 and CVA-24 strains.

	CVA-24	EV-99	CVA-21	EV-96
CVA-24		79.9-87.8	72.1-77.5	72.1-77.0
EV-99	69.4-70.1		72.1-76.2	74.3-78.5
CVA-21	64.0-70.1	64.3-69.8		66.4-70.1
EV-96	63.2-70.2	65.2-69.4	60.9-65.8	

The consensus alignment of these four enterovirus types contained 309 amino acid sites. The aa sites were classified into 5 groups according to pairwise comparisons between EV-96 and the other three types: 1) conserved; 2) polymorphic within and between types; 3) type-specific conservation (i.e., conserved within type but not between types); 4) type-specific polymorphism (i.e., conserved within one type but variable within the other type); and 5) insertion / deletion sites (Table 10).

Table 10. A comparison of the VP1 amino acid sites between EV-96 and CVA-21, EV-99 or CVA-24. The consensus alignment contained a total of 309 amino acids.

	EV-96 vs.		
	CVA-21	**EV-99**	**CVA-24**
Conserved	174	186	184
Polymorphic	18	42	42
Type specific conservation	42	22	18
Type specific polymorphism	64 (45*)	55 (34*)	61 (44*)
Insertion/Deletion	11	4	4

*The sites where amino acid substitution was observed only in a single strand were excluded

The comparison of variable amino acid sites revealed that the polymorphic sites of EV-96 and CVA-21, CVA-24 or EV-99 overlap only partially (Fig. 19). In comparison to EV-99 or CVA-24, the disparities of the polymorphic sites were subtle and often dislocated only by few amino acids, whereas more pronounced differences were observed in comparison to CVA-21. Respectively, the codons with positive selection (detected with the same methods used for EV-96) also overlapped only partially (Table 11). Positive selection was detected at codons 104, 105, 227, 230 and 295 in EV-96; codon 33 in CVA-21; codons 104, 105, 227, 294 and 296 in EV-99; and codons 29, 104 and 105 in CVA-24.

When the acute haemorrhagic conjunctivitis-causing variants of CVA-24 (CVA-24v) were treated as a group separate from the other CVA-24 strains, evidence of positive selection was detected at different sites. For CVA-24v, the only positively selected site was 29, whereas, for the other CVA-24 strains, sites 104, 105 and 294 were under positive selection. Furthermore, several sites that were

polymorphic in EV-96 and the non-AHC-causing CVA-24 strains were conserved in CVA-24v or had polymorphisms only in the earliest isolates. Such sites included amino acids 36, 104, 105, 107, 115, 163, 228 and 304 (Fig. 18).

Many amino acid sites showed a type-specific pattern of conservation (Table 10). These sites included sites where unique type-specific amino acid conservations were detected (i.e., sites where a unique amino acid was conserved in one EV-type and another amino acid in the other EV-type).

Substitutions that change the biochemical properties (e.g., hydrophobicity or the charge) of a corresponding amino acid residue side chain are more likely to change the phenotypic properties (e.g., structure) of the protein. The amino acid sites where such changes were observed between EV-96 and CVA-21, CVA-24 or EV-99 are summarised in Table 12.

Table 11. The VP1 amino acid sites with signs of positive selection. The statistically significant evidence of positive selection is shown in boldface. The significance level (p-value/Bayes Factor/posterior probability) of 0.1 or Bayes factor of 50 (in REL analysis) was used as a confidence limit.

		Normalized dN-dS				Amino acid composition
Type	**Codon**	**HyPhy**	**SLAC**	**FEL**	**REL**	**(n of strains)**
CVA-21	33	1.38	3.11	0.55	**-0.55**	Q (1)
EV-99	104	-0.03	-0.07	0.36	**0.89**	Y (2), G (1), T (4), A (9)
	105	0.47	0.64	4.41	**0.97**	T (4), A (4), S (7), P (1)
	227	0.341	0.43	0.41	**0.94**	V (2), A (8), T (4), D (1), S (1)
	294	0.13	0.10	1.88	**0.98**	Q (10), S (4), N (1), E (1)
	296	2.03	2.84	7.83	**0.98**	T (6), S (5), A (5)
	304	1.03	1.49	0.71	**0.94**	S(3), Q(1), N(6), T(4), A(1), D(1)
CVA-24	29	-0.94	-0.09	0.65	**-0.41**	L (8), H (9), S (1), P (13)
	104	0.04	0.15	0.22	**-0.27**	D (20), T (5), E (6)
	105	-0.27	-0.01	0.36	**-0.28**	A(24), D(2), Q(2), V(1), S(1),T(1)
	294	0.95	0.62	0.23	**-0.30**	Q (26), S (4), A (1)
CVA-24	29	0.17	0.12	-4.41	-1.08	L (7), P (2), H (2)
(non-AHC)	104	1.60	1.56	0.55	**-0.18**	D (2), T (5), E (4)
	105	1.72	1.70	3.57	**-0.21**	A (4), D(2), Q(2), V(1), S(1), T(1)
	294	1.37	1.31	0.45	**-0.20**	Q (7), S (4)
CVA-24v	29	4.41	0.86	**7.32**	-0.11	P (11), H (7), S (1), L (1)
	104	-5.36	-1.01	-10.51	-0.96	D (18), E (2)
	105	0	0	0	-0.84	A (20)
	294	1.21	0.24	4.08	-0.29	Q (19), A (1)

The insertion/deletion sites were at amino acid sites 21-29: CVA-21 had a deletion of 9 amino acids, EV-99 had deletion of 5-6 amino acids and CVA-24 had a deletion of four amino acids compared to EV-96. Very few conserved amino acids were observed between types in this region, whereas only a few singleton amino acid substitutions were detected within the types (with the exception of two EV-99 strains that had an extra amino acid deletion compared to the other EV-99 strains); this finding suggests that the insertions/deletions may have occurred independently for each type. CVA-21 also had a deletion of two amino acids compared to EV-96, EV-99 and CVA-24 at amino acid sites 100-101. At this site the other three types shared a conserved FN sequence.

	STRAIN	29	36	103	104	105	107	115	163	228	294	304
VARIANT	CVA-24-Spain4173EV04	P	L	T	D	A	R	A	L	V	Q	E
	CVA-24- China/GD332/2007	H	L	T	D	A	R	A	L	V	Q	E
	CVA-24-China/GD391/2007	P	L	T	D	A	R	A	L	V	Q	E
	CVA-24-China/GD01/2010	P	L	T	D	A	R	A	L	V	Q	E
	CVA-24-China/GD46/2010	P	L	T	D	A	R	A	L	V	Q	E
	CVA-24-HG	P	L	T	D	A	R	A	L	V	Q	E
	CVA-24-Spain4186EV04	P	L	T	D	A	R	A	L	V	Q	E
	CVA-24-Spain4189EV04	P	L	T	D	A	R	A	L	V	Q	E
	CVA-24-Spain4185EV04	P	L	T	D	A	R	A	L	V	Q	E
	CVA-24-Spain3961O04	P	L	T	D	A	R	A	L	V	Q	E
	CVA-24-Spain4183EV04	P	L	T	D	A	R	A	L	V	Q	E
	CVA-24-Spain4192EV04	P	L	T	D	A	R	A	L	V	Q	E
	CVA-24-03353/SD/CHN/2003/CA24	P	L	T	D	A	R	A	L	V	A	E
	CVA-24-Ningbo3-02	P	L	T	D	A	R	A	L	V	Q	E
	CVA-24-Hangzhou13-02	P	L	T	D	A	R	A	L	V	Q	E
	CVA-24-DSO-26/2005	H	L	T	D	A	R	A	L	V	Q	E
	CVA-24-06.278.4269	H	L	T	D	A	R	A	L	V	Q	E
	CVA-24-SF12Yunnan07	H	L	T	D	A	R	A	L	V	Q	E
	CVA-24-JAM87-10628	S	P	T	E	A	K	A	L	V	Q	E
	CVA-24-variant-Japan(EH24/70)	L	S	T	E	A	K	A	L	V	Q	E
NON-AHC CAUSING	CVA-24-PUR82-10626	P	S	T	T	D	K	P	I	T	Q	N
	CVA-24-PUR82-10686	P	S	S	T	D	K	P	I	T	Q	N
	CVA-24-67897	L	S	T	T	Q	K	P	I	T	S	N
	CVA-24-65902	H	S	T	T	V	K	S	I	T	S	E
	CVA-24-USA-Tx79-10625	L	S	S	E	A	K	P	I	T	Q	D
	CVA-24-E34-dn19	L	S	T	E	S	K	P	I	T	Q	T
	CVA-24-CAM1952	L	P	S	E	A	K	P	I	T	Q	D
	CVA-24-Joseph	L	S	S	E	A	K	P	I	A	S	A
	CVA-24-09088/SD/CHN/2009/CA24	H	S	I	D	T	K	P	I	T	Q	A
	CVA-24-99053/SD/CHN/1999/CA24	L	S	S	T	Q	K	P	I	T	S	K
	CVA-24-05-1	L	S	T	D	A	K	P	I	T	Q	N

Figure 18. The amino acid sites that show evidence of positive selection (circled), or polymorphism among the non-AHC-causing strains of CVA-24 but not the AHC causing CVA-24v strains

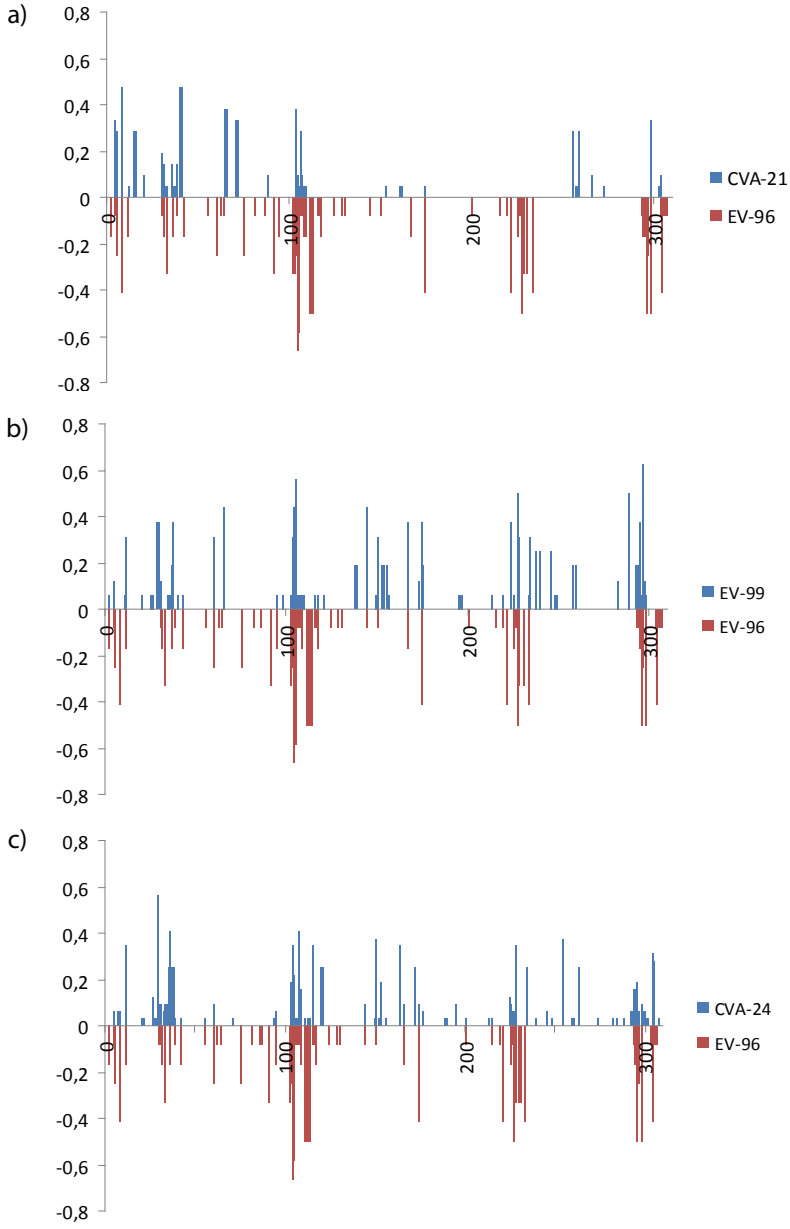


Figure 19. The distribution of the VP1 polymorphic amino acid sites in EV-96 compared to CVA-21 (a), EV-99 (b) and CVA-24 (c). The proportion of polymorphic strains (compared to the consensus sequence) out of the total number of strains in a given type was plotted for each VP1 amino acid site.

Table 12. The amino acid sites that show differences between types in the biochemical properties of amino acid side chains.

Site	Amino acid				Amino acid type			
	EV-96	CVA-21	EV-99	CVA-24	EV-96	CVA-21	EV-99	CVA-24
5	L, I	L, F	V	T	Hydro-phobic	Hydro-phobic	Hydro-phobic	Polar (hydro-philic)
8	T, S	T, S	K	T, A	Polar (hydro-philic)	Polar (hydro-philic)	Positive, highly hydro-philic	Variable
32	A	Q, S	A, S	Q, H, R	Hydro-phobic	Polar (hydro-philic)	Variable	Variable
80	T	C	T	T	Polar (hydro-philic)	SH- (hydro-phobic)	Polar (hydro-philic)	Polar (hydro-philic)
88	S	A	S	S	Polar (hydro-philic)	Hydro-phobic	Polar (hydro-philic)	Polar (hydro-philic)
101	A	S	A	A	Hydro-phobic	Polar (hydro-philic)	Hydro-phobic	Hydro-phobic
110	L	H	Q	Q	Hydro-phobic	Positive highly hydro-philic	Polar	Polar
113	V	T, I	V	T, R	Hydro-phobic	Variable	Hydro-phobic	Variable
115	E, D	N	P, S	P, A	Negative highly hydrophilic	Polar	Variable	Variable
138	T	L	L, I	L	Polar (hydro-philic)	Hydro-phobic	Hydro-phobic	Hydro-phobic
149	Y	P	Y, F	Y, H	Hydro-phobic	Ring*	Hydro-phobic	Variable
155	H	E	H, Y	H, Y	Positive highly hydrophilic	Negative highly hydro-philic	Variable	Variable

Table 12. (continued)

Site	Amino acid				Amino acid type			
	EV-96	CVA-21	EV-99	CVA-24	EV-96	CVA-21	EV-99	CVA-24
175	A	S	A, T	A	Hydro-phobic	Polar (hydro-philic)	Variable	Hydro-phobic
192	T	M	T	T	Polar (hydro-philic)	Hydro-phobic	Polar (hydro-philic)	Polar (hydro-philic)
195	C	N	C, S	S, C, T	SH-group (hydro-phobic)	Polar	Variable	Variable
198	P	P	A	P	Ring*	Ring*	Hydro-phobic	Ring*
237	Q	L	L, Q, S	L	Polar	Hydro-phobic	Variable	Hydro-phobic
238	T	V	V	V	Polar (hydro-philic)	Hydro-phobic	Hydro-phobic	Hydro-phobic
254	H	S	F	F, Y	Positive highly hydrophilic	Polar (hydro-philic)	Hydro-phobic	Hydro-phobic
257	A	H, Y	A, T	A	Hydro-phobic	Variable	Variable	Hydro-phobic
258	R	T	A, R	R	Negative highly hydrophilic	Polar (hydro-philic)	Variable	Negative highly hydro-philic
260	T	H	T	T, I	Polar (hydro-philic)	Positive highly hydro-philic	Polar (hydro-philic)	Variable
261	S	T	A	S	Polar (hydro-philic)	Polar (hydro-philic)	Hydro-phobic	Polar (hydro-philic)
274	V	C	C	C	Hydro-phobic	SH- (hydro-phobic)	SH- (hydro-phobic)	SH- (hydro-phobic)
284	P	L	P	P, S	Ring*	Hydro-phobic	Ring*	Variable

Table 12. (continued)

Site	Amino acid				Amino acid type			
	EV-96	CVA-21	EV-99	CVA-24	EV-96	CVA-21	EV-99	CVA-24
293	K, T	I	K, R, V	K, Q, N, E	Variable	Hydro-phobic	Variable	Variable
296	T, Q, S	A	S, A, T	S, A, T	Variable	Hydro-phobic	Variable	Variable
302	P	K, E	P	A	Ring*	Variable	Ring*	Hydro-phobic
304	T, S, N, A	D	S, Q, N, T, A, D	K, A, D, T, E, N	Variable	Negative highly hydro-philic	Variable	Variable

*The sidechain of proline has a heterocyclic structure. Proline is often found in the bends of protein foldings.

5.4.5 Directional selection: a comparison between types (V)

To detect signs of positive selection between EV-96, CVA-21, CVA-24 and EV-99, the McDonald-Kreitman test (McDonald & Kreitman, 1991) was used. In this method the variable nucleotide sites are classified as fixed (F) (i.e., conserved within type but not between types) or polymorphic (P) (i.e., variable either in one or in both types). The fixed and polymorphic sites are further divided into two groups that contain synonymous (s) and nonsynonymous (n) substitutions. In the case of neutral evolution, the non-synonymous to synonymous substitution ratios should be equal for the fixed and polymorphic sites (i.e., the null hypothesis of the test is E(nF)/E(sF) = E(nP)/E(sP) where E stands for expectation operator). The proportion of base substitutions fixed by natural selection, α, can be estimated in the following way: $\alpha = 1-(sF*nP/nF*sP)$ (Smith & Eyre-Walker, 2002).

The numbers of sites in each class for different EV-types is shown in Table 13. The significance of the difference between the two proportions can be assessed with statistical tests (Fisher's exact test shown here). The McDonald-Kreitman test showed significantly increased proportion of non-synonymous mutations among the fixed sites for inter-typic comparisons, whereas, no deviation from neutrality was observed within EV-96 (Table 13). Although the results of the McDonald-Kreitman test should be interpreted cautiously, since e.g., codon usage bias affects the results (Akashi, 1994), the analysis underlines a strong tendency towards the fixation of different amino acids when different EV-types are compared.

Table 13. The numbers of sites in the McDonald-Kreitman test classes (s=synonymous; n=non-synonymous). P-values were calculated with the Fisher's exact test. α stands for the proportion of fixed base substitutions.

Types compared		Fixed (between groups)		Polymorphic			
		sF	nF	sP	nP	P-value	α
EV-96 (A)	**EV-96 (B)**	18	1	381	48	0.36	-1.268
EV-96 (B1)	**EV-96 (B2)**	20	3	341	39	0.72	0.238
EV-96	**CVA-21**	12	84	448	45	***	0.986
EV-96	**EV-99**	12	43	425	41	***	0.973
EV-96	**CVA-24**	10	42	431	45	***	0.975
CVA-21	**EV-99**	7	50	429	33	***	0.989
CVA-21	**CVA-24**	6	40	439	38	***	0.987
EV-99	**CVA-24**	6	14	415	31	***	0.968

*0.01<P<0.05; ***0.001<P<0.01; ***P<0.001

5.4.6 Amino acid substitutions in relation to the three-dimensional structure of CVA-21 (V)

The inter-typic amino acid substitutions between EV-96 and CVA-21 were superimposed onto the three-dimensional structure of CVA-21 (Xiao et al., 2005). The CVA-21 capsid pentamer structure with amino acid sites classified as indicated in Table 10 is shown in Fig. 20.

Both EV-types have highly polymorphic regions at the buried N-terminal end and at sites 102-105, which are located in the exposed BC-loop close to the star-shaped five-fold axis of the capsid pentamer. Additionally, the insertion/deletion sites were located at these regions.

EV-96 had a highly polymorphic region at sites 225-233 (G-H loop), whereas the amino acid sequence of CVA-21 was completely conserved at this region. Around this region at sites 223, 224, 234, 237 and 238, a type-specific conservation of amino acids was observed, which contained I223L, K224E, Y234F, Q237L and T238V , respectively, for the EV-96 strains and CVA-21 strains (Fig. 22). For CVA-21, these regions (βG and βH strands and the G-H and E-F loops) are among the contact regions between ICAM and VP1 (Xiao et al., 2001, Xiao et al., 2005). In contrast, amino acid sites at the HI-loop showed polymorphism among the CVA-21 strains but not the EV-96 strains.

Many of the EV-96-specific conservations (i.e., polymorphic sites for CVA-21) and inter-typic substitutions that change the biochemical properties(e.g., hydrophobicity) of the homologous amino acid sites were adjacent to the ‘walls’ of the canyon around the star-shaped protrusion at the five-fold axis of the capsid pentamer (Fig. 21).

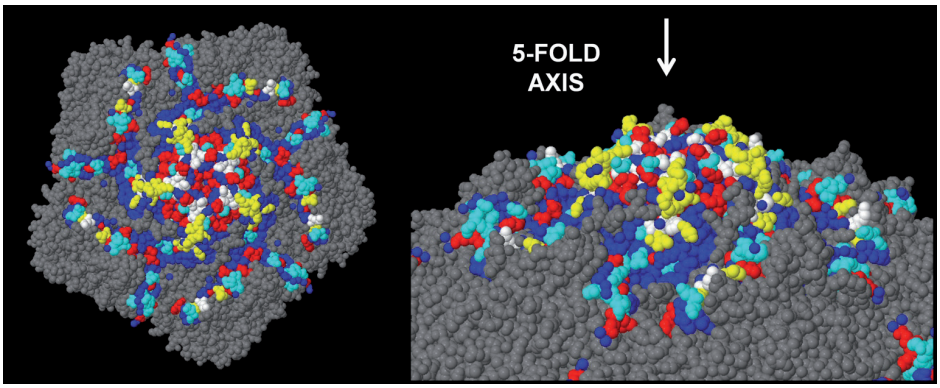


Figure 20. The CVA-21 capsid pentamer; top view (left hand panel) and side view (right hand panel). The VP1 amino acid residues are shown in colour, and VP2-VP4 are shown in grey. The amino acids conserved between the types are shown in blue, the type-specific conservations in red, the polymorphic sites in both types in yellow, the CVA-21-specific polymorphism in white and the EV-96-specific polymorphism in cyan.

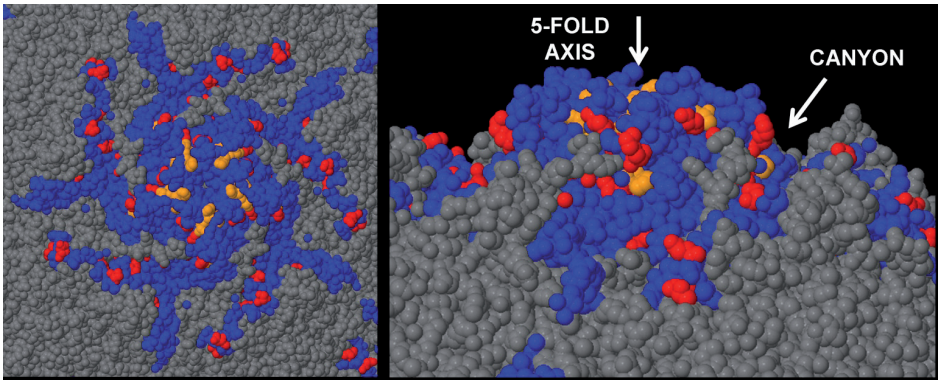


Figure 21. The CVA-21 capsid pentamer: top view (left hand panel) and side view (right hand panel). The VP1 amino acid residues are shown in colour, and VP2-VP4 are shown in grey. The type-specific changes that affect the biochemical properties of the amino acid side chains are shown in red and the EV-96-specific conservations (polymorphic among CVA-21) are shown in orange.

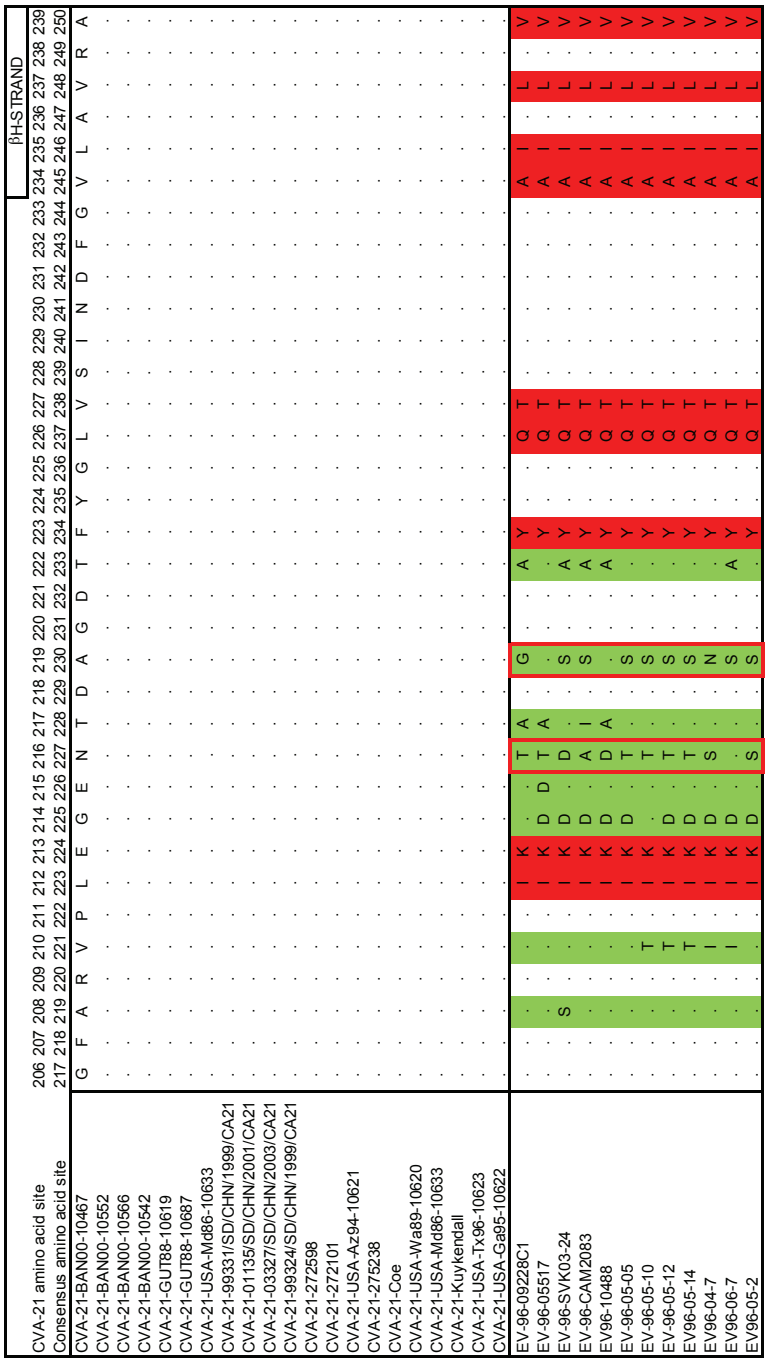


Figure 22. The amino acid sequence alignment that corresponds to the G-H loop and the βH strand involved in CVA-21 receptor binding. The polymorphic sites are indicated in green and the type-specific conservations in red. The amino acid sites with evidence of positive selection are circled in red.

5.5 Phenotypic properties and evolution: HEV-D as a model species (I & IV)

To elucidate the prevalence, time of emergence and possible target tissues (and thus assess the possible clinical symptoms) of new EV-types, the serological and biological properties were studied for EV-94 along with other types of the HEV-D species.

HEV-D is the smallest of the enterovirus species and currently contains only three types: EV-68, EV-70 and EV-94. Enterovirus 68 was first isolated from children with pneumonia and bronchiolitis in California in 1962 (Schieble et al., 1967) and was later shown to include the strains previously classified as human rhinovirus 87 (HRV-87) (Blomqvist et al., 2002, Ishiko et al., 2002). EV-70 was first detected in Ghana in 1969 and has since caused acute haemorrhagic conjunctivitis (AHC) pandemics (Mirkovic et al., 1973). Both of these types are also capable of infecting the central nervous system (Higgins., 1982, Kreuter et al., 2011).

5.5.1 Seroprevalence of HEV-D Types in Finland (I & IV)

The prevalence of antibodies against the HEV-D types in Finland was assessed using the neutralisation assay. Serum samples collected from pregnant women in 1983 (n = 86), 1993 (n = 99), and 2002 (n = 96) were studied. Neutralising antibodies against EV-68 were found in 100% of the subjects, against EV-70 in 15.1%, 21.2%, and 13.5% of the subjects and against EV-94 in 79.1%, 79.8%, and 79.2% of the subjects in the years 1983, 1993, and 2002, respectively (Fig.4 in I, Fig.1 in IV).

The geometric mean end point titres of the sera are shown in Table 1 (in I). The serum samples that were studied had significantly higher levels of antibodies against EV-68 than against EV-70 or EV-94 in all of the years for which samples were studied (Kruskal-Wallis $P < 0.01$). The mean antibody levels against EV-68 showed a decreasing temporal trend, whereas the levels against EV-70 and EV-94 remained constant over the study period (Kruskal-Wallis $P < 0.01$).

5.5.2 Route of infection and tissue tropism (I & IV)

Most enteroviruses use the faecal-oral or respiratory route of transmission. The primary site of infection is usually at the mucosal tissue of the respiratory or gastrointestinal tract. The primary infection can occasionally be followed by a viremic phase. During the viremic phase, the virus spreads through the lymphatic system and circulation and may gain access to the secondary target tissues. The other HEV-D types, EV-68 and EV-70, differ from most enteroviruses by infecting their primary target tissue (the respiratory tract for EV-68 and the conjunctiva for EV-70) directly. The tissue tropism of a virus is a major factor that influences the incidence and severity of enterovirus-induced diseases. Therefore, the possible

route of infection and tissue tropism for the new HEV-D type, EV-94, were studied.

5.5.2.1 Acid sensitivity

Sensitivity to acid treatment and optimum growth temperature infer the primary target tissue and route of transmission of the virus. To access primary replication sites in the alimentary channel mucosa, the virus has to pass through the acidic environment of the stomach. Rhinoviruses, in general, are sensitive to acid treatment, whereas enteroviruses are not sensitive. However, EV-68 (Blomqvist et al., 2002) and some strains of EV-70 have been shown to be sensitive to low pH, and EV-68 has a lower optimum growing temperature than most other enteroviruses (Oberste et al., 2004).

To assess the possible transmission route of EV-94, the acid sensitivity of EV-94 was tested using a standard assay (Couch., 1992). Acid treatment did not affect the infectivities of the environmental EV-94 isolates (Table 3 in I), whereas the titre of the acid-sensitive control, HRV-2, was reduced 10000-fold. The titre of the acid-insensitive control, PV1-Sabin, was not affected by acid treatment.

5.5.2.2 Cell tropism

To assess the cell tropism and potential secondary target tissues of EV-94, viral multiplication and the ability to induce damage in different cell types were studied using continuous cell lines and primary cells.

Consistent with acid stability, EV-94 was able to multiply and induce CPE in colon-derived CACO-2 cells. In addition, several other continuous cell lines were susceptible to EV-94 infection (Fig. 3 in I).

During viremia, blood cells may provide enteroviruses with an additional replication site, thus lengthening the viremia and/or increasing the viral load during viremia. The viruses may also spread to secondary target tissues within circulating leukocytes that have been infected. Therefore, the susceptibilities of monocytic (THP-1, RC-2A, and U937), granulocytic (HL-60 and KG-1), T-cell (MOLT and Jurkat), and B-cell (Raji) lineages to EV-94 and EV-68 infections were studied.

For EV-94, infectious progeny production was detected in all of the studied cell lines 24 h post infection (p.i.) (Fig. 2a in IV). The viability of these cell lines was reduced significantly 3 days after EV-94 infection (ANOVA, $P < 0.005$; Fig. 2b in IV). For EV-68, an increase in viral titre was detected in the T-cell lines MOLT and Jurkat, the B-cell line Raji, the granulocytic cell line KG-1, and in the monocytic cell line U-937, whereas no definitive infective virus production was detected in the monocytic cell line RC-2A or the granulocytic cell line HL-60 (Fig. 2c in IV). EV-68 infection did not induce statistically significant changes in the viability of the leukocytes.

Replication in the endothelial cell lining of blood vessels may provide a virus with an access route to secondary target tissues. EV-94 and EV-70 showed defi-

nite CPE and progeny production in human umbilical vein-derived endothelial cells (Fig. 3a in IV). The infection of endothelial cells was confirmed by the co-staining of the endothelial cell-specific von Willebrand factor and the virus capsid (Fig. 3b in IV). However, there was substantial donor-specific variation in the susceptibility of endothelial cells to HEV-D infections. Infective progeny formation was detected in cells isolated from 12 out of 14 donors after EV-94 infection and 7 out of 11 donors after EV-70 infection. A low level of progeny formation but no definitive cytopathic effect were detected after EV-68 infection in the cells derived from two of the six donors.

The pancreas is one of the most important secondary target tissues for human enteroviruses, and enteroviruses have been considered to play a significant role in the etiology of type 1 diabetes (reviewed in Smura & Roivainen 2012; Hober & Sane, 2011). The pancreatic islets of three donors were infected with both EV-94 and EV-68. Viral infection led to the detection of an infectious progeny formation for both viruses (Fig. 4a in IV). The viability of the islets was drastically reduced 4–7 days after infection with EV-94 (n = 4), whereas the mock-infected control islets and the islets infected with EV-68 (n = 3) remained viable during the same period (Fig. 4b in IV). The co-staining of viral protein and insulin indicated that both viruses were able to infect insulin-producing pancreatic β-cells (Fig. 4c in IV). To assess the β-cell-specific destruction, the insulin to cellular DNA ratios were measured at several time points after infection. The insulin-to-DNA ratio decreased significantly 8–14 days after infection with EV-94 (n = 3). There was no change in the insulin to DNA ratio after EV-68 infection (n = 2) (Fig. 4d).

5.5.2.3 Tropism for non-primate cell lines

Most human enteroviruses have strict host specificity and can infect only human or other primate cells. Accordingly, EV-94 induced CPE and showed definite infectious progeny production in a wide variety of human and other primate cell lines (Fig. 3a in I). However, EV-94 also replicated and induced CPE in a variety of non-primate cell lines such as recombinant mouse L cells expressing human PVR (L20B) and hamster kidney cells (BHK21) (Fig. 3b in I). Virus replication without a strong CPE was found in rabbit kidney cells (RK13). No signs of replication or altered morphology were found in the mouse embryonic fibroblast cell line (3T3). In bovine kidney (MDBK) and canine kidney (MDCK) cells, the virus infection altered cell morphology and induced cell death, but no viable virus progeny formation was detected. However, an enterovirus-specific antibody produced a positive signal in an immunofluorescence assay in MDBK cells (Fig. 3c in I), suggesting that EV-94 is able to internalise into these cells, and there is at least limited viral gene expression. No viral proteins were detected in the MDCK cells.

5.5.2.4 Receptor usage

Viral receptor usage is an important determinant of cell/tissue tropism, host range and pathogenesis. The role of DAF (CD55), the vitronectin receptor (αvβ3) and PVR (CD155) in cell attachment and virus internalisation was studied using CPE protection assays (Nobis et al., 1985). The blocking antibodies were not able to prevent infection in the RD cells.

Chinese hamster ovary (CHO) cell lines expressing either the α2 subunit of the α2β1 integrin or HCAR and mouse fibroblast (M4) cells expressing intracellular adhesion molecule 1 (ICAM-1) were used to assess the role of α2β1, HCAR and ICAM-1, respectively, in EV94 infection. Equal amounts of progeny formation were found in α2- and HCAR-expressing CHO cells. No progeny virus production was found in M4 or M4–ICAM cells, but mild CPE was detected in M4–ICAM cells.

6 Discussion

In this thesis, genetic evidence for the classification of three new enterovirus types was presented. Further characterisation was conducted for one of the new types, enterovirus 94. Sequence analysis methods were used to infer evolutionary relationships between the new and previously known EV types and possible mechanisms behind the emergence of the new enterovirus clades.

6.1 New enterovirus types

The application of molecular typing methods for enterovirus detection has led to the discovery of many new enterovirus types (Table 14) (reviewed in Smura et al., 2011). The enterovirus strains characterised in this thesis formed distinct monophyletic clades within the species HEV-B, HEV-C and HEV-D in their capsid-coding regions and had less than 75% nucleotide sequence (< 85% amino acid sequence) similarities with previously known enteroviruses in their VP1 protein-coding sequences. These results suggest that these strains represent previously unknown enterovirus types and are designated as EV-94 (HEV-D), EV-96 (HEV-C) and EV-97 (HEV-B). The isolates of the new enterovirus types listed in Table 14 have been collected in the past six decades and neutralizing antibodies against one of the new types, EV-94, were detected from serum samples collected three decades ago (I, IV). Furthermore, despite recent discovery, some of the strains (e.g., EV-96) have substantial sequence variation suggesting extensive circulation before the first detection (II, III). Unfortunately, there are currently not enough sequence data to reliably estimate the time of origin for the new enterovirus types described in this thesis (i.e., to conduct molecular clock analysis). The discovery of these apparently new virus types is most likely due to the application of molecular typing methods to samples that have remained ‘untypable’ by antigenic typing methods, direct sequencing from clinical samples and increased typing effort rather than the recent emergence of these viruses in human population. Therefore, the mechanisms behind the appearance of a new EV-type most likely apply also to previously known EV-types.

Table 14. New (i.e. 'numbered') enterovirus types.

	Host species	Year*	Country	Symptoms	Accession no.	Ref.
HEV-A						
EV-71	Homo sapiens	1969	USA / Ca	Encephalitis, Aseptic menigitis; HFMD	U22521	[1]
EV-76	H. sapiens	1991	France	gastroenteritis	AF697458	[2]
EV-89	H. sapiens	2000	Bangladesh	AFP	AF697459	[2]
EV-90	H. sapiens	1999	Bangladesh	AFP	AF697460	[2]
EV-91	H. sapiens	1999	Bangladesh	AFP	AF697461	[2]
EV-92	Macaca mulatta, M.nemestrina, Cercocebus atys	1999	USA	diarrhea	EF667344	[3]
SV-19	M. fascicularis, M. mulatta; M. nemestrina	1956			AF326754	[4]
SV-43	M. fascicularis	1956			AF326761	[4]
SV-46	M. mulatta, M. nemestrina	NA			AF326764	[5]
A-13	Papio cyno-cephalus	NA			AF326750	[6]
HEV-B						
EV-69	H. sapiens	1959	Mexico	None	AY302560	[7]
EV-73	H. sapiens	1955	USA / Ca	NA	AF241359	[8]
EV-74	H. sapiens	1975	USA / Ca	Fever, seizures	AY556057	[9]
EV-75	H. sapiens	1974	Ethiopia	None	AY556065	[9]
EV-77	H. sapiens	1999	France	Meningitis	AY208119	[10]
EV-78	H. sapiens	1999	France	Pneumopathy	AY208120	[10]
EV-79	H. sapiens	1979	USA	NA	AY843297	[11]
EV-80	H. sapiens	1967	USA	NA	AY843298	[11]
EV-81	H. sapiens	1968	USA	NA	AY843299	[11]
EV-82	H. sapiens	1974	USA	NA	AY843300	[11]
EV-83	H. sapiens	1976	USA	NA	AY843301	[11]
EV-84	H. sapiens	2003	Cote d'Ivoire	NA	DQ902712	[11]
EV-85	H. sapiens	2000	Bangladesh	AFP	AY843303	[11]
EV-86	H. sapiens	1999	Bangladesh	AFP	AY843312	[11]
EV-87	H. sapiens	2001	Bangladesh	AFP	AY843305	[11]

EV-88	H. sapiens	2001	Bangladesh	AFP	AY843306	[11]
EV-93	H. sapiens	2000	DRC	AFP	EF127244	[12]
EV-97	H. sapiens	1999	Bangladesh	AFP	AY843307	[11]
EV-98	H. sapiens		Japan	Gastrointestinal	AB426608	[13]
EV-100	H. sapiens	2000	Bangladesh	AFP	DQ902713	[11]
EV-101	H. sapiens	2002	Cote d'Ivoire	NA	AY843308	[11]
EV-107	H. sapiens		Japan	Gastrointestinal	AB426609	[13]
EV-110	Pan troglodytes	2006	Cameroon	NA	JF416934	[14]
HEV-C						
EV-96	H. sapiens	1999	Cambodia	AFP	AB207266	[15]
EV-99	H. sapiens	1984	USA / Ga	NA	EF555644	[16]
EV-102	H. sapiens	1999	Bangladesh	AFP	EF555645	[16]
EV-104	H. sapiens	2004-2007	Switzerland	ARI	EU840733	[17]
EV-109	H. sapiens	2008	Nicaragua	ARI	GU131227	[18]
HEV-D						
EV-68	H. sapiens	1962	USA / Ca	Pneumonia, broncholitis	AY426531	[19]
EV-70	H. sapiens	1969	Ghana	AHC	DQ201177	[20]
EV-94	H. sapiens	2001	DRC	AFP	DQ916376	(I)
EV-111	Pan troglodytes	2006	Cameroon	NA	JF416935	[14]

AFP, acute flaccid paralysis
AHC, acute haemorrhagic conjunctivitis
ARI, acute respiratory infection
HFMD, hand, foot and mouth disease
NA, not available
*Year of the first known isolate

[1] Schmidt et al (1974)
[2] Oberste et al (2005)
[3] Nix et al (2008)
[4] Hoffert et al (1958)
[5] Heberling et al (1965)
[6] Fuentens-Marins et al (1963)
[7] Rosen et al (1973)
[8] Oberste et al (2001)
[9] Oberste et al. (2004)
[10] Norder et al (2003)
[11] Oberste et al (2007)

6.2 How do enteroviruses evolve?

Multiple interconnected processes can be hypothesised to occur during enterovirus evolution. The enterovirus lineages may evolve by gradual diversification (anagenesis) and/or more rapid changes such as adaptive radiation (i.e., a rapid increase in sequence diversity due to a single lineage's adaptation to the environment) or genetic drift during population bottlenecks. As for evolution in gen-

eral, the mechanisms for these processes are mutation and recombination, and the driving forces are natural selection and genetic drift.

6.2.1 Different mutation patterns were observed in intra-typic and inter-typic comparisons

The data presented in this thesis suggests that the evolutionary process leading to the appearance of new enterovirus types may differ from the process behind evolution within intra-typic lineages. The viral VP1-coding sequence of EV-96 was analysed along with the other HEV-C types to gain insight into these processes. Although the data presented here (as with any sequence data set) are bound to represent only a tiny fraction of the actual viral diversity in nature, and the sequence analysis methods used are sensitive to such biases, the high intra-typic sequence diversities detected suggest that the intra-typic datasets can be considered representative samples of the respective viral populations despite their small size.

The dataset contained twelve EV-96 strains with relatively high sequence divergences (approaching the < 25 % nt divergence limit for grouping into the same type). The phylogenetic analysis (II, III) suggested that all 12 of the strains group robustly (i.e., with high bootstrap support) into two major clusters. The larger of the clusters (Cluster B) also contained subclusters of more closely related strains.

Despite robust clustering by nucleotide sequences, no cluster-specific amino acid signatures could be detected for EV-96 (with the exception of phylogenetically very close relatives, which are likely to share similar point mutations by genetic drift). These results suggest that the genetic diversity and clustering within the EV-96 type is largely due to silent mutations.

The selection analysis detected strong negative selection at most of the sites and relieved negative selection or positive selection at a few highly polymorphic sites. This detection may be explained by a selective pressure posed by the host immune system to amino acids at the virus surface (e.g., antigenic sites). Mutations at these sites could allow the virus to escape from the host immune response. Due to the adaptability of the host immune system, the advantage could be short term, because the circulation of the virus in a host population results in an increase in the proportion of immune hosts, which changes the ‘direction’ of the selective pressure. Consequently, the novelty of the site (for the host population) is most likely more important for viral fitness than fixation of a distinct amino acid sequence. At the virus population level, this changing selective pressure could result in a highly polymorphic site.

Alternatively, amino acid toggling (i.e., switching between a wild type amino acid that is associated with high replicative fitness and susceptibility to the immune response and an escape state with lower replicative fitness) is expected for a

class of amino acid sites that mediate escape from host immune system at the cost of replicative fitness for the virus (Delport et al., 2008). Toggling has been suggested to occur during, for example, EV-71 circulation (Tee et al., 2010). The large number of singleton amino acid mutations among the EV-96 strains suggests that deviations from a wild type sequence are continuously produced. Although none of the EV-96 VP1 amino acid sites were detected as toggling, parallel amino acid substitutions were detected at several sites. Therefore, the failure to detect toggling sites may be due to the small sequence dataset.

These results suggest that the intra-typic evolution of the EV-96 VP1 protein is most likely dominated by (mostly neutral) genetic drift combined with selection at antigenic sites even though different EV-96 clusters have a significant degree of divergence in both nucleotide and amino acid sequences. For most of the codons, negative selection was detected, which suggests a strong evolutionary pressure to retain the amino acid sequence and, thus, structure of VP1. Intra-typic lineages would therefore have similar structural constraints with variation occurring predominantly at specific sites, including antigenic sites and their proximity, the loops between β-sheets and the structurally disordered amino-terminal segment of VP1 (as was suggested for polioviruses (Jorba et al., 2008, Kinnunen et al., 1990)).

This hypothesis is also supported by the analyses of other enterovirus types. Intra-typic sequence comparisons of enteroviruses have revealed generally low non-synonymous to synonymous substitution frequency ratios (dN/dS or Ka/Ks) (Bailly et al., 2009, Jorba et al., 2008, Simmonds, 2006, Suzuki, 2004, Tee et al., 2010). For polioviruses, almost all of the amino acid sites of the capsid proteins were found to be under negative selection, suggesting that the evolution of the neutralising antigenic sites had occurred primarily by genetic drift (Jorba et al., 2008, Suzuki, 2004). For other enteroviruses, positive selection maintaining amino acid polymorphism has been detected only at a few distinctive positions presumably at the outer surface of the VP1 capsid proteins of E-30 and EV-71 (Bailly et al., 2009, Tee et al., 2010).

Intra-typic evolution by antigenic drift (i.e., change of antigenic sites) that most likely resulted from selection and/or genetic drift at restricted antigenic sites has probably occurred also with EV-68, where the majority of amino acid substitutions occur at potentially antigenic VP1 BC- and DE-loops (Meijer et al., 2012). This evolution is in line with the declining temporal trend of geometric mean titres of neutralising antibodies against the EV-68 prototype strain Fermon (IV).

In contrast to the intra-typic evolution of EV-96, the inter-typic comparisons between EV-96 and its closest relatives CVA-21, EV-99 and CVA-24 suggest that mere antigenic changes are not sufficient to explain the differences between the EV types. The polymorphic sites and sites that may be evolving under positive selection overlap only partially between different EV types. Furthermore, in con-

trast to the intra-typic comparisons between genetic clusters, the type-specific amino acid signature sites can be easily identified. These observations suggest that structural changes have occurred during divergent evolution of EV-types in addition to changes at antigenic sites. A structural change could expose new amino acid sites to selective pressure imposed by the host immune system and, on the other hand, require fixation at sites where a polymorphism was previously allowed.

Accordingly, many of the amino acid differences between types may potentially change the structure of the VP1 protein. For example, these substitutions include those that change the hydrophobicity of a given amino acid site. Although these substitutions can be also detected between strains of a single EV-type, substitutions within a type are almost uniformly located at highly polymorphic sites (and therefore most likely in structurally disordered regions, such as loops between β-sheets) or found only in a single strain.

6.2.2 Possible mechanisms for the emergence of new enterovirus types

The inter-typic comparisons outline the differences between the types but do not explain the appearance of the differences (i.e., the origin of serotypes). One possibility is that the divergence between intra-typic lineages gradually increases, and current phylogenetic patterns are explained by the extinction of 'transitional forms' and/or gaps in sequence data. Such transitional forms between enterovirus types have not been detected. When the pairwise VP1 sequence similarities between the EV-strains are plotted, intra-typic and inter-typic similarity values form clearly separated groups rather than a continuum between the serotypes (Brown et al., 2009, Oberste et al., 1999, Oberste et al., 1999). Furthermore, the observed evolutionary pattern of the capsid-coding region with largely negative codon-specific selection and a few sites under relaxed negative selection or positive selection (maintaining polymorphism) suggests strict constraints for enterovirus capsid evolution.

Such constrictions might be posed by, for example, the structural demands of the capsid-coding proteins in which variation might be expected only at specific sites. In contrast, the diversity between serotypes is not restricted to individual amino acid sites or protein domains but affects the whole capsid-coding region, suggesting that the diversification of a serotype has required a profound reorganisation of the capsid proteins (Simmonds, 2006).

6.2.2.1 Niche changes

The reorganisation of the capsid structure may be linked to viral adaptation to a new ecological niche. In this scenario, intra-typic polymorphism normally occurs within the limitations imposed by a given niche (e.g., structural restrictions of the capsid, receptor usage, and temperature and acid sensitivity), and the changes beyond the 'accepted' variation most likely decrease the fitness of a virus. Never-

theless, due to heterogeneous nature of enterovirus populations such fitness decreasing mutations are continuously generated.

The high mutation frequency of RNA viruses provides enormous potential for adaptation (Domingo et al., 2008). In this respect, highly prevalent singleton amino acid mutations that were detected among the EV-96 strains might be considered as evolutionary experiments that, on occasion, may enable the colonisation of a new environment or adaptation to a changing environment. Most of these mutations, however, are not likely to increase the fitness of the virus in a given environment and will not become fixed in the virus population.

Experimental studies suggest that very small changes in the viral capsid proteins may affect viral cell type-specificity, tissue tropism, host species, cytopathogenicity, host cell response to infection and the immune response (Al-Hello et al., 2005, Al-Hello et al., 2009, Arita et al., 2008, Caggana et al., 1993, Cameron-Wilson et al., 1998, Chua et al., 2008, Cifuente et al., 2011, Colston & Racaniello, 1995, Halim & Ramsingh, 2000, Kim & Racaniello, 2007, Knowlton et al., 1996, Pan et al., 2011, Pelletier et al., 1998, Polacek et al., 2005, Ramsingh & Collins, 1995, Ramsingh et al., 1997, Schmidtke et al., 2000). Changes like these are well within the reach of rapidly mutating RNA viruses. In this scenario, a viral subpopulation with an altered phenotype (e.g., receptor binding affinity, receptor usage, a new co-receptor, an alternative receptor, evasion of the host immune system, cell/tissue tropism, host cell replication/translation machinery usage, etc.) might be able to occupy a new niche. A new niche would likely have different selection pressures (different fitness or adaptive landscape) than the previous niche, forcing further mutations to increase fitness. Furthermore, the occupation of a new niche would present a population bottle-neck, where some mutations would most likely become fixed by chance. The transitional (low fitness) forms would be quickly outcompeted and extinct (and most likely not detected). Consequently, the adaptation of the virus to the new niche would be detected as an emergence of a new virus type.

A single mutation may also have a pleiotropic effect on virus functions (i.e. affecting many functions). Accordingly, there is evidence of coevolution of cell recognition and antigenicity in picornaviruses (Baranowski et al., 2001). For many viruses, there is an overlap between receptor binding sites and antigenic sites. For example, the residues of the poliovirus antigenic sites influence viral receptor recognition (Harber et al., 1995, Murray et al., 1988). Thus, antibody-directed selection might indirectly affect the evolution of receptor tropism, and vice versa.

Intriguingly, several type-specific amino acid differences between EV-96 and CVA-21 mapped close to the known receptor interaction sites, suggesting that altered receptor binding may have been involved in the divergent evolution of these types. The receptor usage of EV-96 has not been studied, but CVA-21 shares the ICAM-1 binding property with many of the other HEV-C types, including

CVA-13, CVA-15, CVA-18 and CVA-20 (Newcombe et al., 2003). However, ICAM-1-binding HEV-C types also have significant differences in cell attachment because, despite a common receptor usage, amino acid residues that represent the ICAM-1 footprint are not conserved among these types (Newcombe et al., 2003). Furthermore, there are apparent differences in co-receptor usage because CVA-21 also uses DAF for attachment and membrane concentration (Newcombe et al., 2004, Shafren et al., 1997), whereas CVA-13 binds to an unidentified co-receptor, and CVA-20 uses an unidentified internalisation receptor (Newcombe et al., 2003). These differences suggest considerable plasticity in the receptor/co-receptor usage of HEV-C viruses – another feature prompting adaptation. In addition to receptor binding, the large differences in the N-terminal region of VP1 between EV-96 and CVA-21 suggest that subsequent events in viral uncoating (such as extrusion of the N-terminus of VP1 and the release of VP4 (Couderc et al., 1993, Moss & Racaniello, 1991)) may be different between these enterovirus types.

CVA-24, a close relative of EV-96, provides an example of evolution through adaptation to a new target tissue. The AHC-causing variant of CVA-24 (CVA-24v) that emerged in 1970 in Singapore (Lim., 1973) forms a subcluster within the CVA-24 group (Fig. 23). The phylogenetic analyses suggest a single origin for all of the AHC-causing variants (Fig. 23) (Brown et al., 2009). Antigenically CVA-24 variants are considered to be prime strains of CVA-24 (i.e., the CVA-24v strains are poorly neutralised by the antiserum that neutralises the prototype strain, but the antiserum made against the variant strains are able to neutralise the isolate and the prototype) (Brown et al., 2009, Mirkovic et al., 1974). CVA-24v uses sialic acid as a receptor (Nilsson et al., 2008), but the receptor for the non-AHC-causing CVA-24 strains is not known. Remarkably, another AHC-causing enterovirus type, EV-70, uses the same receptor (Alexander & Dimock., 2002, Nokhbeh et al., 2005). Thus, a receptor switch (possibly) from ICAM-1 to sialic acid might have changed the tissue tropism of the ancestor of CVA-24v from the gastro-intestinal tract to conjunctiva and therefore enabled the virus to occupy a new niche.

Despite a considerable amount of conservation in the nucleotide and amino acid sequences, different evolutionary processes may be operating for CVA-24v and the non-AHC-causing CVA-24 strains at several sites. Intriguingly, the sites under positive selection appear to differ between CVA-24v and other CVA-24 strains. For example, at sites 105 and 304, strong polymorphism was detected for the non-AHC causing CVA-24 strains, whereas the CVA-24v strains showed complete conservation at these sites. At some sites (e.g., 36, 104, 107 and 254), the earliest sequenced isolates of CVA-24v resembled the non-AHC causing CVA-24 strains, whereas the fixation of a different amino acid apparently occurred thereafter at the CVA-24v lineage. These results suggest that CVA-24v might still be under ongoing adaptation, ultimately leading to a new EV type. Accord-

ingly, the phylogenetic analyses suggest a ladder-like tree with a chronological trend for CVA-24v (Chu et al., 2009). Alternatively, some of the amino acid fixations among the CVA-24v strains might be due to genetic drift during the presumably tight population bottle-neck experienced by the first CVA-24 strain capable of infecting conjunctiva.

The different CVA-24v lineages can be efficiently separated by the nonstructural protein-coding sequences (Chu et al., 2009), suggesting that AHC-causing variants of CVA-24 do not recombine with other EVs, which is possibly due to different cell tropism. Due to the recombination barrier, the AHC-causing variants of CVA-24 are likely to evolve independently of other CVA-24 strains either by genetic drift or adaptive evolution.

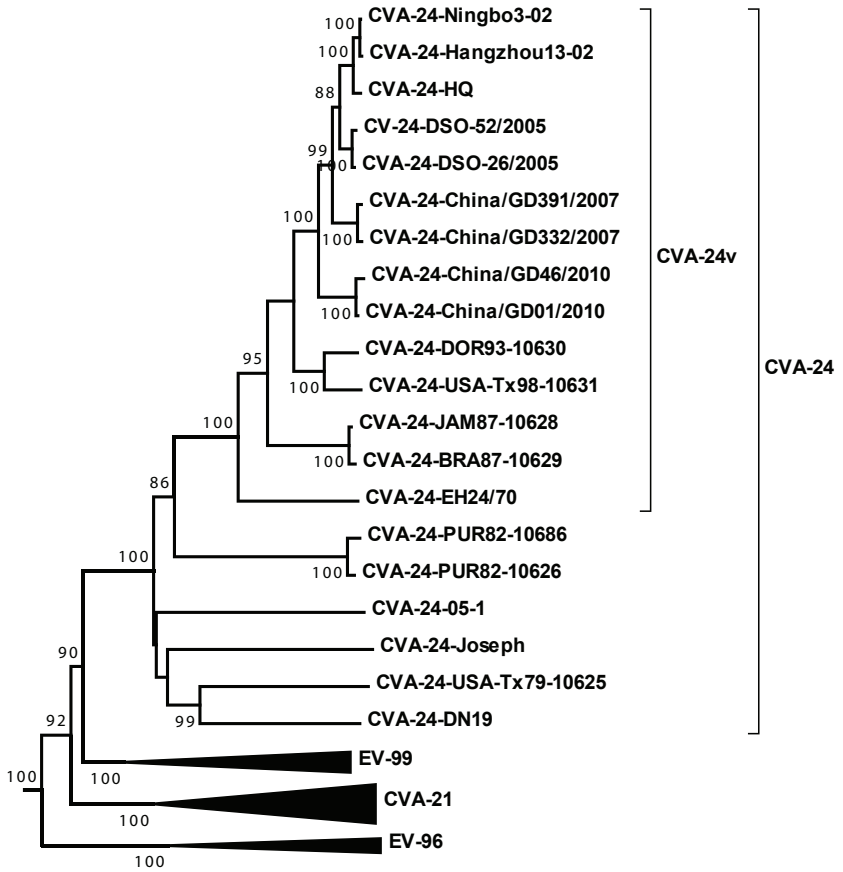


Figure 23. A phylogenetic tree showing clustering of the CVA-24 AHC-causing variants (CVA-24v) and the non-AHC causing CVA-24 strains. The tree was constructed from the P1 coding sequences using the Neighbour-Joining method of tree construction. The genetic distances were computed using the Tamura-Nei model for nucleotide substitutions. The bootstrap support values were calculated for 1000 replicates.

6.2.2.2 Inter-species transmission

Another way to change niches is spreading from one host species to another. Recent studies (Harvala et al., 2011, Harvala et al., 2012, Oberste et al., 2005) have suggested that several enterovirus types that represent three of the four known HEV species (HEV-A, HEV-B and HEV-D) can circulate in various non-human primate species.

It has also been speculated that Enterovirus 70 (EV-70), a member of the HEV-D species, might have a zoonotic origin. EV-70 was discovered in Ghana in 1969 during an acute haemorrhagic conjunctivitis epidemic (Mirkovic et al., 1973) and has subsequently caused large-scale epidemics around the world. It has been estimated that all EV-70 isolates have a common ancestor that emerged in one location in 1967±15 months (Miyamura et al., 1986, Takeda et al., 1994). Among the enteroviruses, EV-70 has an unusually wide host range *in vitro*. EV-70 is able to replicate in cells derived from a wide range of mammalian species (Yoshii et al., 1977), and neutralising antibodies against EV-70 have been detected in the sera of cattle, sheep, swine, chickens, goats, dogs and monkeys (Kono et al., 1981, Sasagawa et al., 1982). However, EV-70 isolates have not been found in animals, and the presence of neutralising antibodies against EV-70 in animals might be due to an antigenic cross-reaction between EV-70 and some (unknown) animal virus.

Similar to EV-70, EV-94 shows an unusually wide cell tropism *in vitro* (I, IV), suggesting that it might also be capable for cross-species transmission. A recent seroprevalence study revealed that, in addition to European human populations (I, IV), a high prevalence of antibodies against EV-94 is present in the human populations of sub-Saharan Africa (85 %, 68 % and 80 % in Cameroon, Zimbabwe and South Africa, respectively), whereas lower seroprevalences were detected among chimpanzees and various Old World monkeys (13 % and 2 %, respectively) (Harvala et al., 2012). Although the primary host species for EV-94 seems to be human, these results suggest that non-human primate populations are also exposed to this enterovirus type. Intriguingly, in the same study, high prevalences of antibodies against E-11 and EV-76 were found among chimpanzees, gorillas and various Old World monkeys providing evidence for inter-species exposure to human enteroviruses (Harvala et al., 2012).

The HEV-D serotypes have other features unusual to enteroviruses. EV-68 and some strains of EV-70 are acid labile (Blomqvist et al., 2002, Oberste et al., 2004), whereas EV-94 is acid stable similar to other enterovirus serotypes (I). EV-68 is also temperature sensitive. These features most likely reflect the tissue tropism of these viruses. EV-68 (including strains previously classified as HRV-87) is a respiratory tract pathogen (Blomqvist et al., 2002, Ishiko et al., 2002, Oberste et al., 2004, Schieble et al., 1967), and the primary infection site of EV-70 is at conjunctival cells (Mirkovic et al., 1973). Both of these viruses can occasionally infect the central nervous system (Higgins., 1982, Kreuter et al., 2011). EV-94, on the other

hand, has been isolated from the stool samples of patients with acute flaccid paralysis (I) (Junttila et al., 2007) and is able to replicate in various potential target cells *in vitro* including neuroblasts, leukocytes, endothelial cells and pancreatic islets (I, IV). EV-94 most likely uses the faecal-oral route of transmission (I), whereas EV-68 and EV-70 are transmitted primarily *via* direct or indirect contact with respiratory, oropharyngeal or conjunctival secretions (Pallansch & Roos., 2001).

The unique features of the HEV-D types pose a question about the origin of this species. If EV-70 emerged from an animal host, where did EV-68 and EV-94 emerge from? EV-68 was first isolated 5 years prior to the estimated emergence time of EV-70. EV-94 was discovered recently, but the serological studies (I, IV) suggest that this virus has been circulating at least since the 1980s. Relatively high genetic divergences and a lack of recombination between the three HEV-D serotypes suggest an independent evolution of these viruses, and the divergence of these three types from their common ancestor most likely occurred long before the detection of the first isolates. If EV-70 indeed emerged from an animal host, EV-68 and EV-94 either emerged independently from an animal host or, alternatively, the members of HEV-D species circulate in multiple host species.

Recently, a fourth HEV-D type (EV-111) was discovered in the wild chimpanzee population in Cameroon (Harvala et al., 2011). Intriguingly, this type apparently circulates in both chimpanzee and human populations. The chimpanzee-derived strain bears substantial similarity to a HEV-D strain (previously classified as EV-70) isolated from a stool sample from a child with AFP in the Democratic Republic of Congo (Harvala et al., 2011, Junttila et al., 2007). EV-111 clusters with EV-70 in the VP1 region and EV-94 in the 5'UTR and 3D region. These phylogenetic inconsistencies suggest that EV-70 and EV-111 share a common ancestor in the capsid-coding region, whereas, in the other genomic regions, EV-94 and EV-111 may have recombined. Notably, the complete genome analysis (I) provided no evidence for recombination between EV-68, EV-70 and EV-94. The ancestor of EV-111 and EV-70 may have been a gastrointestinal pathogen, and (analogous to CVA-24 and CVA-24v) the change of tissue tropism from intestinal mucosa to conjunctiva created a recombination barrier between EV-70 and EV-111/EV-94 and promoted divergent evolution of EV-111 and EV-70.

6.2.2.3 Inter-species transmission & Capsid-coding sequence evolution

CBV-5/Swine vesicular disease virus (SVDV) provides an example of capsid-coding sequence evolution during the adaptation process following host species switching. SVDV originated from CBV-5 as the virus spread from humans to pigs (Brown et al., 1973, Graves., 1973, Zhang et al., 1993, Zhang et al., 1999) with an estimated inter-species transfer time between 1945 and 1965 (Zhang et al., 1999). The Bayesian evolutionary analysis of the VP1-coding region suggested that,

among the CBV-5 lineages, the evolutionary rate has been highest for the branch leading to the most recent SVDV lineages (Gullberg et al., 2010).

Both CBV-5 and the older SVDV strains (isolated in the 1950s and 1960s) use the coxsackie-adenovirus receptor (CAR) as a primary receptor and are able to use human decay-accelerating factor (DAF) as a co-receptor (Bergelson et al., 1997, Martino et al., 2000). However, CBV-5 does not bind to pig DAF but infects porcine cells via pig CAR (Spiller et al., 2002). The more recent SVDV strains (isolated in the 1990s) have lost their ability to bind human DAF and instead use heparin sulphate for attachment in porcine epithelial cells (Escribano-Romero et al., 2004, Jimenez-Clavero et al., 2005). The comparative structural and amino acid sequence studies of CBV-5 and SVDV capsids suggest that adaptation to a new host species has induced changes in the CAR-binding site (partially overlapping the putative DAF binding site), the putative heparan sulphate-binding site, the antigenic sites, the VP1 hydrophobic pocket and around the five-fold axis of the capsid (Fry et al., 2003, Verdaguer et al., 2003). Some of the more recent SVDV strains also contain block deletions in the 5'NCR between the end of IRES and the translation initiation site (Shaw et al., 2005).

The apparent similarity in the changes occurring during the adaptation of CBV-5 to a new host species to those that have occurred during the divergent evolution of EV-96 and CVA-21 (i.e., changes in receptor binding sites, putative antigenic sites and around the five-fold axis of the capsid) suggests similar changes in selective pressures during the colonisation of a novel niche and formation of two distinct enterovirus types from a common ancestor. However, despite the rapid colonization of a new niche (similar to CVA-24v), SVDV is not (yet) divergent enough to merit classification into a new enterovirus type. Furthermore, separation of CVA-24v and SVDV into new types would leave CVB-5 and CVA-24 as paraphyletic clades. This scenario suggests that either adaptation to a new niche does not inevitably lead to the formation of a new type, or alternatively, the process may take a relatively long time. Nevertheless, the formation of a new enterovirus type may involve a selection period during which adaptive mutations are fixed and a more neutral period during which polymorphism increases.

6.2.2.4 The role of recombination in adaptation to a new niche

The host species most likely has a significant influence also on the evolution of viral non-capsid sequences due to specific interactions between host and viral proteins or host proteins and viral RNA (Oberste et al., 2007). The phylogenetic analysis of the 5'UTR region (II) provides insight into evolutionary processes that may also be involved in the inter-species transmission of viruses. In this region, human enteroviruses are grouped into three clusters: one cluster consists of the 'old' HEV-C and HEV-D types, another cluster consists of HEV-A and HEV-B types and the third cluster consists of two recently described HEV-C types, EV-104 and EV-109. This clustering suggests that at least two inter-species recombi-

nation events have occurred: one between the ancestors of the HEV-A and HEV-B species and another between the ancestors of the HEV-C and HEV-D species (Santti et al., 1999). Between the modern HEV strains, however, inter-species recombination seems to occur only rarely (Smura et al manuscript).

In addition to human enteroviruses the HEV-A species contains viruses (SV-19, SV-43, SV-46, BaEV and EV-92,) that infect other primates (*Macaca mulatta, M. nemestrina, M. fascicularis, Cercocebus atys and Papio cynocephalus*) (Oberste et al., 2002, Oberste et al., 2007, Oberste et al., 2008). In phylogenetic trees the 5'UTRs of these virus types do not cluster together with those of the HEV-A types that infect humans but form several outgroups to the HEV-A/HEV-B cluster (II). However, in the capsid-coding region, the currently known simian HEV-A types cluster together with the recently described HEV-A serotypes that infect humans, EV-76, EV-89, EV-90 and EV-91. These results suggest that inter-species transmission occurs from humans to other primates or *vice versa*. Correspondingly, an EV-76 strain was recently detected from a stool sample collected from the wild chimpanzee population in Cameroon (Harvala et al., 2011).

The 5'UTR sequences of EV-76 and EV-89 group together with the HEV-A/HEV-B cluster, whereas those of EV-90 and EV-91 group together with the HEV-C/HEV-D cluster (II). These results suggest that an inter-species recombination event occurred between the ancestor of EV-90/EV-91 and a member of the HEV-C or HEV-D species. Because the 5'UTR contains IRES secondary structures that bind to host cell-specific translation factors, this region may be especially important for the colonisation of a new host species. Therefore, the inter-species recombination event between EV-90/EV-91 and a HEV-C/HEV-D strain may be an adaptation to a new host species (i.e., humans). A shift from the 'monkey-specific 5'UTR' to the 'human-specific 5'UTR' may have provided the recombinant virus with a selection advantage over the original virus. Correspondingly, the ancestor of EV-76/EV-89 may have recombined with a member of HEV-A or HEV-B in the 5'UTR or alternatively gained mutations convergent to those in the HEV-A/HEV-B cluster (II).

In experimental settings, inter-species 5'UTR recombinants are generally viable (Gromeier et al., 1996, Rohll et al., 1994, Schibler et al., 2012, Todd et al., 1997) but often have lower fitness than the parental viruses (Schibler et al., 2012). It is likely, therefore, that inter-species recombinants are able to outcompete the parental virus only when the parental virus has low fitness in the given host cell – a situation which can be expected to occur during host species switching.

Curiously, EV-76 and EV-89 form an outgroup to the HEV-A/HEV-B 5'UTR cluster (II) and EV-76, EV-89, EV-90 and EV-91 form a distinct group in the non-structural protein-coding regions (P2 and P3) of the genome, indicating recombination within this group of novel HEV-A types but not between this group and other HEV-A viruses (Oberste et al., 2005). These results suggest that EV-76, EV-

89, EV-90 and EV-91 might occupy a unique ecological niche, forming a recombination barrier between the 'new' and 'old' HEV-A types.

Inter-species recombination (or extensive evolution independent of enteroviruses that infect humans) may have also occurred with the Baboon enterovirus (BaEV), which clusters together with the HEV-A strains in the P1 region only and is not closely related to the HEV-A viruses when other genomic regions are analysed (Oberste et al., 2007). In this case the inter-species recombination may have been an adaptation for replication in baboon cells.

6.3 The role of recombination in enterovirus evolution

Enteroviruses, in general, have high rates of recombination within and between types (Simmonds & Welch, 2006, reviewed in Lukashev, 2005). As discussed above, recombination may aid virus adaptation to new or changing environments by providing large genome segments that are possibly pre-adapted to the new niche. Furthermore, recombination may permit the elimination of harmful mutations (see the paragraphs on Muller's ratchet in the Introduction, page 29) or adoption of advantageous traits. Accordingly, recombination events often precede the emergence of novel EV lineages (McWilliam Leitch et al., 2009, McWilliam Leitch et al., 2010, McWilliam Leitch et al., 2012, Mirand et al., 2007, van der Sanden et al., 2011). However, it has proven difficult to demonstrate the benefits of recombination experimentally (Pliaka et al., 2010, Savolainen-Kopra et al., 2009a, Schibler et al., 2012).

The frequency of inter-typic recombination differs between HEV species: the frequency is the highest within the HEV-B species and lower within the HEV-A and HEV-C species (Simmonds & Welch, 2006). Within the HEV-D species, no evidence of inter-typic recombination was detected (I). However, minor inconsistencies in the clustering pattern of EV-111 in the VP1, 5'UTR and 3D suggest that recombination may have occurred between EV-94 and EV-111 (Harvala et al., 2011). These results suggest that the impact and constraints for recombination may differ between EV species and types.

Several factors restrict recombination. These factors include viral prevalence and cell tropism, sufficient nucleotide similarity, co-operation of cis-acting elements within the genome, the functionality of the resulting chimeric proteins and the co-operation of mature proteins. The co-infection of a certain cell is a prerequisite for recombination, and different tissue tropism might efficiently restrict recombination. As discussed above, these restrictions might occur between members of the HEV-D species, because the three serotypes of this species have different primary replication sites and cell tropism (I, IV). Similarly, there seems to be a recombination barrier between the conjunctiva tropic CVA-24v and other HEV-C strains. Furthermore, the CVA-21 strains that have a propensity to cause respiratory tract infections tend to cluster together at P2 (Fig. 8b) and the 5'UTR (Smura

et al., manuscript). However, in the case of CVA-21, the recombination barrier is incomplete, because not all of the strains cluster together in the P3 region (Fig. 8c), suggesting infrequent and possibly ancient recombination events between CVA-21 and other HEV-C types.

Sequence similarity-derived restrictions for recombination may be exemplified by the clustering pattern of the HEV-C strains (III, V). The clustering pattern suggests frequent inter-typic recombination at the 3' end of the genome. However, the detected recombination events become less frequent towards the 5' end of the genome because, in the P2 region, recombination was detected mainly between the strains that are closely related in the P1 region. These strains formed a clustering pattern where deep nodes of the tree were generally congruent to those of the P1 tree (with the exception of CVA-13/18 and some EV-96 strains), but more recent type-specific nodes were not. At the 5'UTR, however, the P1-specific clustering breaks down again. A similar trend can be observed in the recombination history of EV-97: promiscuous recombination occurred at the 3' end of the genome, but no recombination partners were found for the majority of the P2 sequence.

With no assumptions about the mechanism or preferred sites of recombination, the probability of a recombination event should increase with the genomic linkage distance, i.e., the genomic sites that are physically close to one another are likely to be inherited together. This scenario would propose gradually increasing recombination frequency from P1 towards the 5' and 3'ends of the genome.

Because homologous recombination requires substantial sequence similarity between recombination partners (Worobey & Holmes, 1999), recombination is expected to be more common between close relatives and become gradually less common with more divergent strains. Furthermore, if nucleotide similarity increases recombination frequency, a positive feedback effect, where a recombination event increases the probability of further recombinations, would be expected.

The clustering pattern of the HEV-C strains in different regions of the genome suggests that another force is affecting recombination frequencies. With the HEV-C strains, recombination occurs preferentially within the major clusters, but genetic distance appears not to have a strong effect within major clusters. This scenario would result in a stepwise increase in the frequency of recombination reflecting virus phylogeny.

The combined effects of linkage, sequence similarity and phylogeny in addition to the pre-requisition of similar cell tropism may explain the observed recombination pattern with preferred recombination partners. In this scenario, the concordance of the deep (ancient) nodes of P1 and P2 trees would be a result of preferred recombination within the clade. The two exceptions to major phylogenetic clusters (A-C) in the P2 region, CVA-13 and EV-96, suggest that the phylogeny barrier can be crossed (i.e., there is no strict incompatibility between the P1 and P2 regions of cluster A, B and C). Apparently, crossing the barrier occurs infre-

quently because CVA-13 and EV-96 remain as separate clusters (with further subclustering) within clusters A and B at the P2 region, suggesting that the recombination event is most likely ancient. In the P3 region, the linkage effect would partly outweight the similarity and phylogeny effects, resulting in an increase in observed phylogeny violations towards the 3' end of the genome. The 5'UTR does not show signs of the linkage effect (the 5'UTR is physically close to the P1 region, but there is no similarity to the P1 grouping). However, this region is highly conserved and provides enough sequence similarity for frequent recombination. Therefore this region is functioning as a 'recombination hot spot', where recombination events occur at a higher frequency than at other regions.

Altogether, the phylogenetic pattern of the HEV-C strains suggests non-random recombination between strains of the same species. Therefore, the frequency of recombination appears to differ between species (Simmonds & Welch, 2006), types and intra-species groupings, which is possibly due to cell tropism, sequence similarity and the phylogenetic position of a given virus strain.

7 Concluding remarks and future prospects

A large number of novel enterovirus types have been characterised during the past decade, raising a question about the origin of the new viruses. In this thesis, three new enterovirus types were established by sequence properties, and one of the new types (EV-94) was subjected to further characterisation of phenotypic properties. Furthermore, genetic evidence of the general patterns of enterovirus evolution, which ultimately leads to the emergence of new virus types, was presented.

The discovery of many new enterovirus types reflects the application of molecular typing methods and an increased typing effort rather than the recent emergence of these viruses in the human population. Almost all of the proposed new types are characterised only by sequence; hardly anything is known about the biology of the proposed new types (e.g., receptor usage, tissue/cell tropism, host responses, disease associations and epidemiology). Some of the new serotypes (including EV-96 and EV-97) have been detected several times during the last few years, suggesting high prevalence and wide geographic circulation. For the others, not enough data exists to draw conclusions about the prevalence and epidemiology of the virus. The sample types, clinical associations and geographical origins of the proposed new types show similarity to the previously established serotypes i.e., there seem to be respiratory and gastrointestinal pathogens among the new enterovirus types, and many of the types may be capable of causing neurological symptoms.

The sequence analysis presented in this thesis suggests different modes of evolution within enterovirus types (resulting in intra-typic lineages) and during evolution, which leads to larger scale (type-specific) differences. In this respect, intra-typic genetic change would be dominated by silent mutations accompanied by amino acid polymorphism occurring dominantly at immunogenic sites. This genetic change can be observed as a high rate of synonymous mutations, strong negative selection and amino acid polymorphism and/or positive selection at distinct sites in structurally disordered regions. Inter-typic differences, on the other hand, are most likely accompanied by structural alterations in capsid proteins, which cause changes in the pattern of the conserved and polymorphic amino acid sites and could be a result of adaption to larger scale changes in the environment. Intra-typic variation would therefore occur in a restricted sequence ‘space’ that retains the capsid structure. On occasion, a novel variant could, however, jump

from one sequence space (or fitness landscape) to another, manifesting itself as an emergence of a new EV-type.

The observations described above suggest that a taxonomical level equivalent to serotype (or enterovirus type) is still useful even though historically a 'serotype' was defined by antigenic characteristics of a virus and molecular methods have surrogated the neutralization tests. The differences between types are not solely the ones at antigenic sites. Instead, these differences reflect more profound structural changes between the serotypes (and therefore the antigenicity or serotype of a virus merely reflects more profound differences between viruses). Thus, it appears that the antigenic properties and the phylogenetic position (i.e., the serotype or the type) of each enterovirus reflect a specific ecological niche and/or other profound differences in the viral structure. Therefore, the empirical limits (the '75/85 % rule') for enterovirus typing seem to have a solid evolutionary and biological basis.

Altogether, these observations suggest that both adaptation and immunoselection (combined with structural changes) may be required for the formation of a new type. Furthermore, there is no need to assume that similar processes are behind the formation of each new EV-type because the events that predispose the formation of a new type are stochastic (and viruses are opportunistic). Recombination most likely has a dual role in this process by providing gene flow between viruses with similar cell tropism and enough sequence similarity (therefore homogenising the virus population) and creating new combinations of genetic traits. Therefore recombination increases the plasticity of a virus population and possibly facilitates adaptation to new niches by providing genes pre-adapted to a certain niche.

Evolutionary inferences are dependent on the amount of reliable sequence data. However, in respect of enteroviruses, sequence data have accumulated for a relatively short period. In addition, there are both geographical and clinical sampling biases, which hamper the evolutionary analyses. As more sensitive detection methods that do not require virus culture in cells and/or previous knowledge about viral sequence become more widely used and previously understudied sample types (e.g., environmental samples or archived samples) are studied, previously unknown picornavirus types, species and genera will be characterised in humans and other animals. This is likely to revolutionise enterovirus taxonomy and deepen our understanding of enterovirus evolution. Furthermore, a more thorough understanding of population dynamics, epidemiology and pathogenesis of enteroviruses can be established.

8 Acknowledgements

This work was carried out at the Virology Unit of the Department of Infectious Disease Surveillance and Control, National Institute for Health and Welfare, Helsinki, Finland.

I am grateful to my supervisor Merja Roivainen for guidance and encouragement throughout this project and, most of all, giving me the opportunity to study these exciting new viruses.

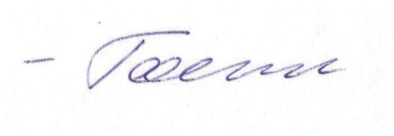
I wish to express my gratitude to the reviewers Prof. Alexander Gorbalenya and Doc. Alexander Plyusnin for their valuable and encouraging comments on the thesis.

The experts in viral evolution, Prof. Emer. Tapani Hovi, Dr. Soile Blomqvist and Dr. Carita Savolainen-Kopra are thanked for sharing their wisdom in discussions on evolution and other more or less related subjects.

I warmly thank the present and former members of the lab: Haider al-Hello, Mervi Eskelinen, Päivi Hirttiö, Svetlana Kaijalainen, Alena Kaijalainen, Päivi Klemola, Marja-Liisa Ollonen, Anja Paananen, Eija Penttilä, Johanna Rintamäki, Marja-Leena Simonen-Tikka and Petri Ylipaasto for creating great atmosphere and helping me out innumerable times during this project.

Special thanks to my family and friends - and especially to my wife Noona -for their love and support, and, of course, to Potti, Martta and late Pietari for not giving a damn about science but taking me out to the forests and fields.

This work has been financially supported by Finnish Cultural Foundation, University of Helsinki, European Union, Sigrid Juselius Foundation, Juvenile Diabetes Research Foundation and Academy of Finland.



Helsinki 29.6.2012

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